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In vitro study of the impacts of exposure to mixtures of endocrine disrupting chemicals on the aryl hydrocarbon and steroid receptor transcriptional activity

By

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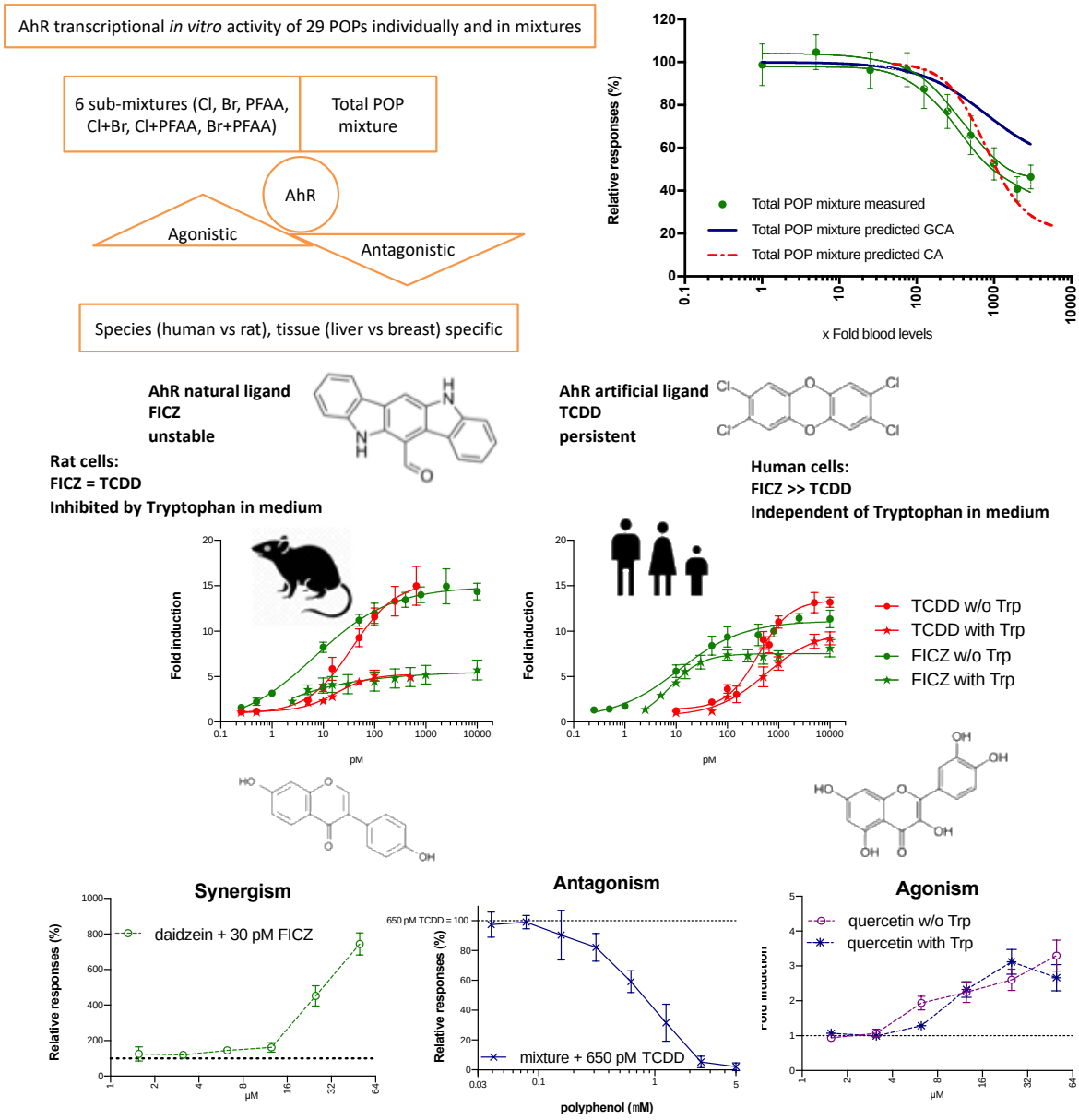
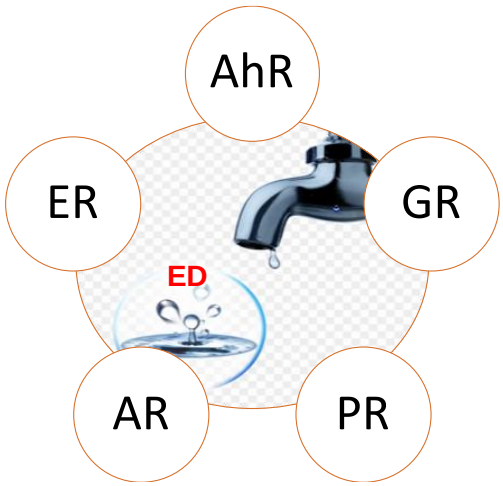
Abstract

Chemicals are undoubtedly important and beneficial for our modern life. As a result, we are exposed to mixtures of chemicals in our daily life through applying them for food production and preservation and for supporting human and animal health and recreation. However, risk assessment for the consumer is usually based on a chemical-by-chemical approach. Among these chemicals, endocrine disruptors (EDs) are of concern, in particular because they are able to alter the function(s) of the endocrine system, leading to adverse health effects in organism or (sub) population levels. *In vitro* Chemically Activated LUciferase gene eXpression (CALUX) assays involving several transgenic reporter cell lines are interesting tools to study the impacts of exposure to mixtures of EDs and their components on the transcriptional activity of the master xenobiotic receptor, which is the aryl hydrocarbon receptor (AhR), as well as the steroid (estrogen (ER), androgen (AR), progesterone (PR), and glucocorticoid (GR)) receptors. Three mixtures were being investigated as examples of compound groups of human everyday exposure to chemicals: (1) the "total POP mixture" consisting of 29 POPs (persistent organic pollutants) prevalent in Scandinavian human blood, (2) the "ED mixture" containing 18 potential EDs dominantly found in Wallonia raw water intended for drinking water production, and (3) the "polyphenol mixture" containing seven food-based polyphenols. The concentration of each component in the mixture was based on human-relevant exposure such as fold human blood level (the POP mixture), fold maximum quantified concentration in raw water (the ED mixture), or fold recommended intake dose from food supplements (the polyphenol mixture). Specific aims of the project were: (a) evaluating species (rat and human) and/or tissue-specific (hepatocytes and mammary gland) AhR responses to the POP mixture and the polyphenol mixture and their components, (b) profiling the endocrine disrupting activities of the EDs and the mixture thereof prevalent in raw water using AhR and steroid receptors, (c) identifying interactions among the chemicals (additive, antagonistic or synergic effects) on the transcriptional activity of the receptors, (d) identifying the actual chemical(s) the most active in the mixtures, and (e) predicting the effect of the mixtures based on the activity of single compounds. The results showed that 16 out of 29 POPs contaminating human blood were AhR antagonists. The total POP mixture also showed an AhR antagonistic activity although it contained each compound at the concentration below its lowest-observed-effect concentration (LOEC). Chlorinated compounds were the drivers of the activity of the total POP mixture, among which PCB-118 and PCB-138 contributed for 90% of the total POP mixture effect. From the 18 EDs prevalent in raw water, chlorpyrifos, bisphenol A, fluoranthene, phenanthrene, and benzo(a)pyrene demonstrated significant activities on several receptors. Noticeably, benzo(a)pyrene mixed with dioxin TCCD induced a synergistic response in AhR-

reporter human mammary gland cells (DR-T47-D), 10-fold higher than the cells' response to TCDD alone, at a concentration which could be a realistic blood level after a food contamination incident or in a high exposed sub-population. The mixture of the 18 EDs compounds exerted AhR and ER agonistic activities, which can be explained by the activities of benzo(a)pyrene and bisphenol A in the mixture. While the rat AhR reporter cells (DR-H4IIE) was more sensitive to POP exposure, we showed for the first time that the AhR endogenous ligand FICZ, a tryptophan derivative was more potent than TCDD in the human AhR (DR-HepG2) (40 times more potent than TCDD) while both exhibited a similar potency in the rat cells (after 6h exposure). Two isoflavones (daidzein and genistein) induced a higher AhR agonistic/synergistic activity in the rat cells, while the others (a flavonol (quercetin) and two flavones (baicalin and chrysin), curcumin, and the mixture of the seven polyphenols) caused a stronger AhR antagonistic response in the human cells. Quercetin and resveratrol were the strongest AhR antagonists in the human cells, which contributed most for the antagonistic activity of the polyphenol mixture. Dose-response curves were predicted successfully by concentration addition and general concentration addition models for the POP mixture, while both concentration addition and independent action performed well for estimating the effect of the polyphenol mixture, indicating the additive activity of the components in these mixtures. The results suggested that the endocrine disrupting activities of chemicals in human daily life exposure could involve more than one mechanism: their (anta-) agonistic effects on different receptors with the potential for additive, inhibitory or synergistic effects of mixtures thereof should be considered in risk assessment.

Graphic abstracts





List of Abbreviations

AhR: aryl hydrocarbon receptor

AHRR: AhR repressor

AIP: AhR-interacting protein

AR: androgen receptor

ARNT: aryl hydrocarbon receptor nuclear translocator

BaP: benzo[a]pyrene

BDE: brominated diphenyl ether

BFRs: brominated compounds

bHLH: Helix-Loop-Helix

BPA: Bisphenol A

Br: brominated compounds

CA: concentration addition

CALUX: Chemical-Activated LUciferase gene eXpression

Cl: chlorinated compounds

CYP1A1: cytochromes P450 1A1

CYPs: cytochromes P450

DDE: dichlorodiphenyldichloroethane

Dex: dexamethasone

DHT: 5-α-dihydrotestosterone

dl-: dioxin like

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethylsulfoxide

DNA-binding domain (DBD)

DR: dioxin responsive

DREs: dioxin responsive elements

E2: 17β-estradiol

EC_{50}: half maximal effective concentration

EDs: endocrine disruptors

ER-MMV: human estrogen receptor transgenic cells

ER: estrogen receptor

EREs: estrogen response elements

EROD assays

FBS: fetal bovine serum

FICZ: 6-formylindolo[3,2-b]carbazole

GCA: generalized concentration addition

GR: glucocorticoid receptor

GREs: glucocorticoid response elements

H4IIE: rat hepatoma cells

HBCD: hexabromocyclododecane

HCB: hexachlorobenzene

HCH: hexachlorocyclohexane

HepG2: human hepatoma cells

Hsp90: heat shock protein 90

IA: independent action

IC_{50}: half maximal inhibitory concentration

IF: induction fold

KO: knockout

LBD: ligand-binding domain

LBP: ligand binding pocket

LDH: Lactate dehydrogenase

LOECs: lowest observed effect concentrations

MCF-7: human breast cancer cell

MEMα: Minimum Essential Medium

MMTV: Mouse Mammary Tumour Virus

MOAs: mode of actions

MOECs: maximum observed effect concentrations

MR: mineralocorticoid receptor;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NOECs: No Observed Effect Concentrations

NOELs: No Observable Effect Levels

NRs: nuclear receptors

OCPs: organochlorine pesticides

P23: co-chaperone phosphoprotein

P4: progesterone

PAHs: polycyclic aromatic hydrocarbons

PAS: Per-Arnt-Sim

PBDEs: polybrominated biphenyl ethers

PCBs: polychlorinated biphenyls

PCDDs: polychlorinated dibenzodioxins

PCDFs: polychlorinated dibenzofurans

PFAAs: perfluorinated compounds

PFCs: perfluorinated compounds

PFCs: surfactant perfluorinated compounds

PFDA: perfluorodecanoic acid

PFHpA: perfluoroheptanoic acid

PFHxA: perfluorohexanoic acid

PFHxS: perfluorohexanesulfonic acid

PFNA: perfluorononanoic acid

PFOA: perfluorooctanoic acid

PFOS: perfluorooctanesulfonic acid

PFUnDA: perfluoroundecanoic acid

POPs: persistent organic pollutants

PR-TM: human progesterone receptor transgenic cells

PR: progesterone receptor

PRB: progestin receptor isoform B

RB: retinoblastoma

REPs: relative effect potencies

RLUs: relative light units

RPC_{Max}: efficacy

RR: relative response

SCPOP: Stockholm Convention on Persistent Organic Pollutants

SRs: steroid receptors

T47-D: human mammary gland cells

TARM: human androgen receptor transgenic cells

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TEFs: toxic equivalency factors

TEQs: toxic equivalent quantities

TGF-β: transforming growth factor beta 1

TGRM: human glucocorticoid receptor transgenic cells

Trp: tryptophan

XAP-2: hepatitis B Virus X-associated protein 2

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Chapter 1: Introduction

1. Human are exposed to mixtures of chemicals.

Chemicals indispensably support our daily life and sustain our civilization. However, some chemicals can cause deleterious effects on our health and our living environment. With thousands of chemicals in the market and an accelerating production rate, more and more chemicals are used or even overused worldwide for improving quality of life (*e.g.* food production, supplies of goods and services). These currently used chemicals, together with already historical chemicals, are ubiquitous in the environment nowadays and become unavoidable in daily life. Indeed, humans are chronically exposed to multiple exogenous substances, directly through intended applications and uptakes of chemicals in personal care products or pharmaceuticals or food nutrients, or indirectly from contaminated food and feed or pollutants in water and air. The mixtures act in concert simultaneously in human bodies, possibly resulting in adverse health effects.

1.1. Persistent organic pollutants

Toxic chemicals can be persistent organic pollutants (POPs). During the industrialization after World War II, thousands of these synthetic chemicals were introduced into commercial use for pest and disease control, crop production, and industry. However, these compounds have unforeseen adverse effects on human and environment health[1,2].

POPs are typically halogenated (chlorinated, brominated, and fluorinated) organic compounds with high lipid solubility, high resistance to degradation and poor metabolization. They, therefore, are able to accumulate in the fatty tissues of living organisms for a long period of time, and they are susceptible to biomagnification and long-range transportation[3–5]. As a result, high trophic predatory, long-lived, and lipid-rich organisms (*e.g.*, fish, marine mammals, and humans) can have POP accumulation at hazardous levels in their body[6]. Indeed, POPs can be detected at measurable concentrations virtually everywhere on our planet even in regions where they were never used[2]. For example, their capacity of transportation in the atmosphere and water, when adsorbed to particles, enables them to contaminate secondary sources even in remote areas, such as the Arctic[7]. The commonly detected POPs are persistent organochlorine pesticides (OCPs) applied intensively for agricultural production, polychlorinated biphenyls (PCBs) as industrial chemicals, flame retardant polybrominated biphenyl ethers (PBDEs) and surfactant perfluorinated compounds (PFCs) for improving product characteristics, as well as the unintentional by-products of many industrial processes,

especially polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF), commonly known as 'dioxins'[8].

A global treaty, the Stockholm Convention on Persistent Organic Pollutants (SCPOP), entered in force in 2004, aims at reducing and ultimately eliminating production, use, trade, release, and storage of POPs to protect humans and the environment from their adverse effects. Although the production and use of POPs have been generally stopped, POPs still remain as a global issue, currently posing threats to humans and wildlife due to their “already” ubiquitous distribution in the living organisms and the environment[8]. They have been detected in almost every human sample, including fetuses, embryos[9], and human fluids[10,11], and also are found frequently in feed[12] and food[13], including for example tuna[14], Atlantic Salmon[15], seals[16], humpback dolphins[17], dugongs[18], *etc*. Exposure to POPs can lead to homeostatic disorders, cardiovascular diseases[19,20], and metabolic diseases such as obesity and diabetes[21–23]. Some food-borne POPs cause cancers and hypospadias for fetal and infant males, breast cancer, cystic ovaries, and endometriosis for females[24]. Among the POPs, dioxins, PCBs, PBDEs, PFCs and OCPs, typically contaminating human bodies will be briefly discussed.

Dioxins (PCDDs and PCDFs) and dioxin-like PCBs (dl-PCBs) belong to the highest toxicity group, with their general chemical structures shown in Figure 1. Among them, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been classified in group 1 of carcinogenic compounds to humans (IARC, International Agency for Research on Cancer). Dioxins and dl-compounds are persistent, ubiquitous and toxic byproducts of various industrial, combustion, and natural processes such as waste incineration, home heating, and the production of organic chemicals containing chlorines such as OCPs and PCBs. Humans are exposed to dioxins mainly (more than 90%) via meat and fish consumption, where fish is the primary source[25,26,27].

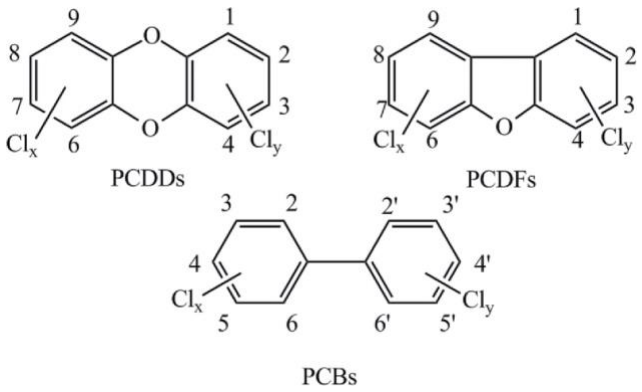

Figure 1: General chemical structures of PCDDs, PCDFs, and PCBs. The possible positions of chlorine atoms on the benzene rings are designated by numbering the carbon atoms[37].

Dioxins exert toxic effects even at very low concentrations. The lethal doses cause slow death as a result of a wasting syndrome, while nonlethal doses cause reproductive and developmental effects, tumor promotion, immunity suppression, and skin lesions[28]. Several "dioxin" crises have been recorded in the past years, such as contaminations through the Agent Orange used by the United States during the war in Vietnam (1962–1971)[29], a mass food poisoning termed "Yusho" due to PCDFs in Japan in 1968[30], a severe industrial accident in Seveso, Italy, in 1976, releasing up to kilograms of dioxins into the air[31], and the notorious "Belgian" dioxin crisis which resulted from an episode of feed contamination in 1999[32–34]. TCDD cannot be rapidly metabolized and thereby inactivated by detoxification enzymes, such as cytochrome p450 (CYPs) enzymes[35,36]. TCDD in humans and organisms can last for years, with half-lives on average of 1.6 years for persons < 18 years old, and 3.2 years for those ≥ 18 years of age[36].

Table 1: Dl-PCBs, PCDDs and PCDFs and non dl-PCBs levels in human milk, in several countries: arithmetic means and minimum and maximum within brackets[38].

Subregion	PCBs-PCDDs-PCDFs in WHO TEQ units (pg/g lipids)	PCBs-PCDDs-PCDFs TEQ threshold (pg/g lipids)	PCBs (ng/g lipids)	PCBs threshold (ng/g lipids)
eastern Europe[1]	13 (5.9–19)	0.2	152 (34–376)	0.7
western Europe[2]	14 (10–19)		136 (69–221)	
northern Europe[3]	8.9 (6.7–12)		63 (24–90)	
southern Europe[4]	15 (10–20)		210 (136–252)	
western Asia[5]	7.4 (5.8–8.9)		28 (27–29)	
central Asia[6]	7.3		20	

1 Bulgaria, Czech Republic, Hungary, Republic of Moldova, Romania, Russian Federation, Slovakia, Ukraine
2 Belgium, Germany, Luxembourg, Netherlands, Switzerland
3 Finland, Ireland, Lithuania, Norway, Sweden
4 Croatia, Italy, Spain
5 Georgia, Cyprus
6 Tajikistan

Source: data from WHO & UNEP (2013:27–28)

Occurring normally at higher concentration than dioxins and dl-compounds, other groups of chlorinated compounds including non dl-PCBs and OCPs are also found in human bodies (Table 1)[39]. PCBs were introduced in the 1880s and came into commercial use in 1929, while OCPs appeared between the 1940s and 1950s. Most of their production was banned in the late 1970s and 1980s due to their adverse health effects. Even at low doses of exposure, they can adversely affect health and normal biological function, especially during disease stages

such as cancer[40] or during development stages such as childhood or pregnancy[41]. Since their ban, the levels of most POPs have been declining over time, however, the levels of PCBs and dl-compounds in human milk are still one to two orders of magnitude higher than the threshold values proposed by WHO (Table 1)[38].

PCBs were widely used as dielectric and coolant fluids in electrical devices, carbonless copy paper, and in heat transfer fluids[42]. Although no longer in use, PCBs may still leak from old equipment and building materials[43]. The insecticide dichlorodiphenyltrichloroethane (DDT) and γ-HCH/lindane (γ-hexachlorocyclohexane) are among those OCPs still in limited use, mainly as a low-cost solution for malaria control[44,45]. DDT and its main environmental degradation product, dichloro-diphenyldichloroethylene (DDE)[46] are persistent and bioaccumulative compounds that are highly soluble in lipid and found commonly in human bodies[38]. Several OCPs such as hexachlorobenzene (HCB), α-HCH, and β-HCH are by-products of the manufacturing of other chemicals. Meanwhile, chlordanes, aldrin, and dieldrin have been banned in Europe and the United States, but are still being used in Asian and African countries[47]. Their isoforms, α(*cis*)- and γ(*trans*)-chlordane, *trans*-nonachlor[48], and their metabolites *oxy*-chlordane[49] and dieldrin are currently detected in human samples. The structure of several typical OCPs are shown in Figure 2.

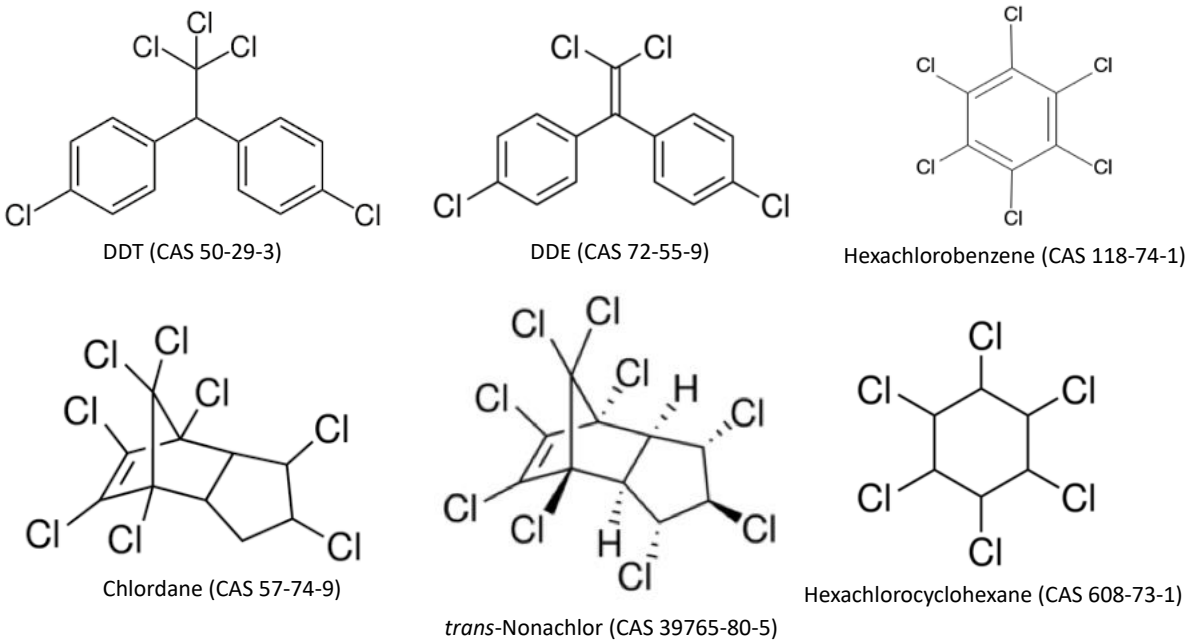

Figure 2: Examples of OCPs listed in SCPOPs.

There is increasing evidence showing the link between health problems and exposure to PCBs and OCPs. For example, an increased mortality risk in humans was associated with higher

levels of highly chlorinated PCBs, especially for cardiovascular diseases[50]. Meanwhile, high levels of OCPs resulted in increased non-cancer, non-heart/cerebrovascular disease in US elderly[51]. Indeed, OCPs can alter the endocrine system and cause adverse effects on the reproductive, neurological and immune systems, increasing the risk of developing hormone-dependent cancers and affecting sexual differentiation, growth and development[52–54].

Flame retardants introduced since the 1960s, such as PBDEs, have been applied in textiles, electrical and electronic products[55]. Figure 3 illustrates several common structures of the flame retardants. There are three commercial mixtures of congeners with different levels of bromination namely: penta-BDE (dominated by BDE-47, BDE-99 and BDE-100), octa-BDE (dominated by BDE-183, BDE-153 and BDE-154) and deca-BDE (dominated by BDE-209). The other brominated compound, hexabromocyclododecane (HBCD) has been used in polystyrene industry[56]. PBDEs have been detected in increasing levels in human (breast milk) and wildlife samples[56]. Exposure to these chemicals can lead to endocrine disruption[56–58], developmental neurotoxicity[59], and delayed mental and psychomotor development in infants due to maternal exposure[60]. The three commercial PBDE mixtures and HBCD are currently listed under SCPOP.

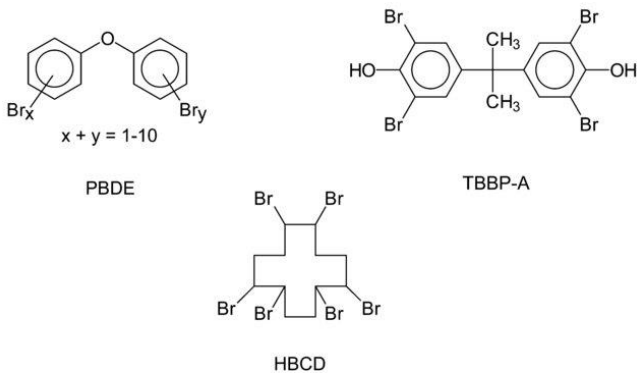

Figure 3: Chemical structures of PBDEs, tetrabromobisphenol A (TBBP-A), and HBCD[61].

The perfluorinated compounds (PFCs), which were first synthesized in the 1940s–1950s, have received increasing attention since the early 2000s due to their frequent detection in human and environmental samples[62,63] and their associated health concerns. In a Scandinavian study, they have been shown to be the most abundant group contaminating human blood[11]. They consist of a hydrophobic alkyl chain of various length (typically from 4 carbon atoms (C4) to 16 (C16)) and a charged/neutral hydrophilic end group that is fully fluorinated, resulting in their amphiphilic character (Figure 4). Due to their many useful properties such as chemical inertia, non-wetting, very slippery, nonstick, highly fire resistant, very high temperature ratings, and highly weather resistant, they have been applied widely in fluoropolymer-coated

cookware, sports clothing, extreme weather resistant military uniforms, food handling equipment, medical equipment, motor oil additives, fire-fighting foams, paint and ink as well as water-repellent products[38].

Exposure to PFCs can lead to metabolism disorders, immunotoxicity, potential carcinogenicity[64], possible neurodevelopmental defects[65], risk of miscarriage[66], and cancer in humans[67]. Currently, only perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) and their salts are listed under SCPOP. Meanwhile, other PFCs such as perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA) as well as perfluorohexane sulfonic acid (PFHxS) are also highly persistent with long half-lives in humans (Figure 4)[62,68,69].

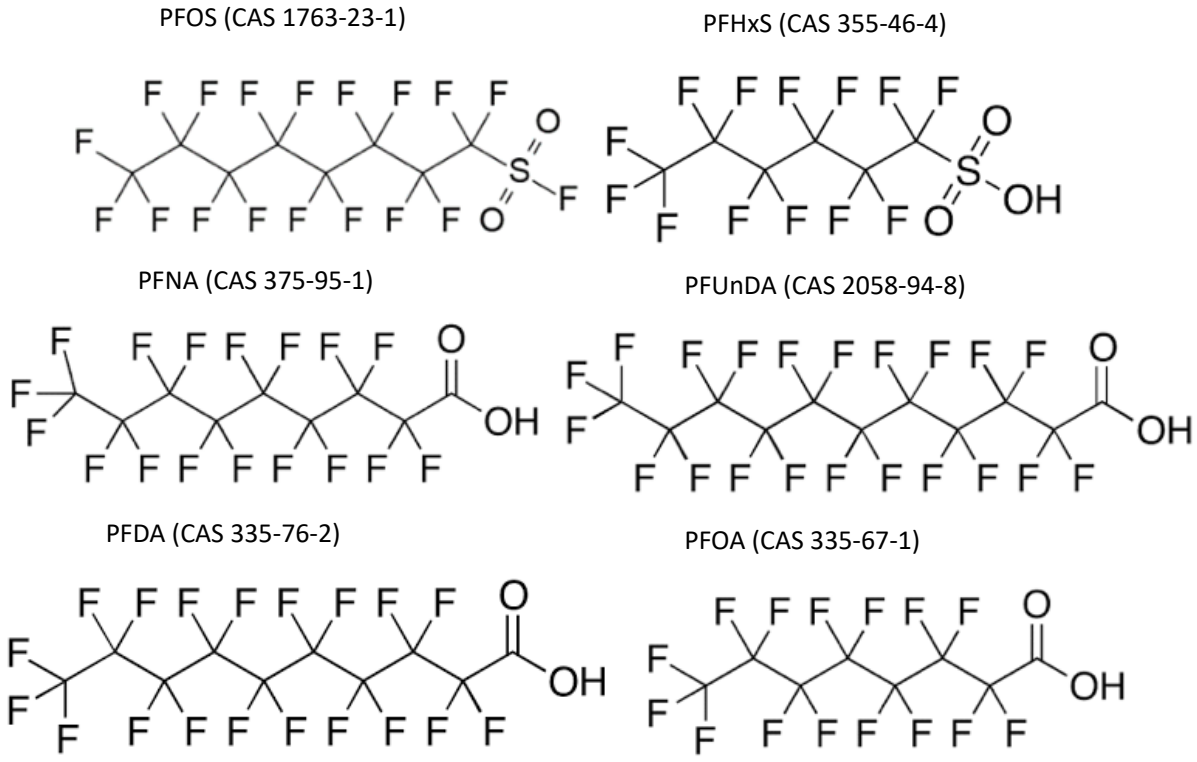

Figure 4: Structures of PFOS, PFAS and other related PFCs.

1.2. Non-persistent organic pollutants

Non-persistent organic pollutants belong to the emerging group of hazardous chemicals. These chemicals are readily decomposed in the environment and rapidly metabolized in the body. However, owing to their toxicity, they can cause harmful effects on human health and the environment. Among non-persistent organic pollutants, polycyclic aromatic hydrocarbons (PAHs), non-persistent pesticides and herbicides, and bisphenol A (BPA), typically contaminating water sources will be briefly discussed.

PAHs are generated during incomplete combustion of organic materials found in wood, tobacco smoke, smog, ambient air pollution and charcoal-grilled and overcooked meats. They are considered as the most potent carcinogens[70]. PAHs, such as benzo(a)pyrene (BaP), are rapidly metabolized and thereby quickly eliminated from the body. Due to their lipophilic nature, many PAHs undergo the full biotransformation, *i.e.* enhancing polarity (phase I), conjugating to hydrophilic moieties (phase II), and eliminating across the cell membrane (phase III)[71]. For example, the metabolite of BaP, BaP-7,8-diol-9,10-epoxide which is able to form DNA-adducts, is highly mutagenic (genotoxic), considered as the "ultimate carcinogenic" derivative[72]. It is the product of hydroxylation of BaP by CYP1 isoenzymes, preferentially CYP1A1. Hence, the generation of highly genotoxic metabolites by CYP1A1-mediated oxidation of BaP and related PAHs depends on the capacity of the phase II enzyme system[73–75]. However, the dose, route and duration of exposure significantly influences the detoxifying or toxifying action of CYP1A1. The structures of BaP, fluoranthene, and phenanthrene are shown in Figure 5.

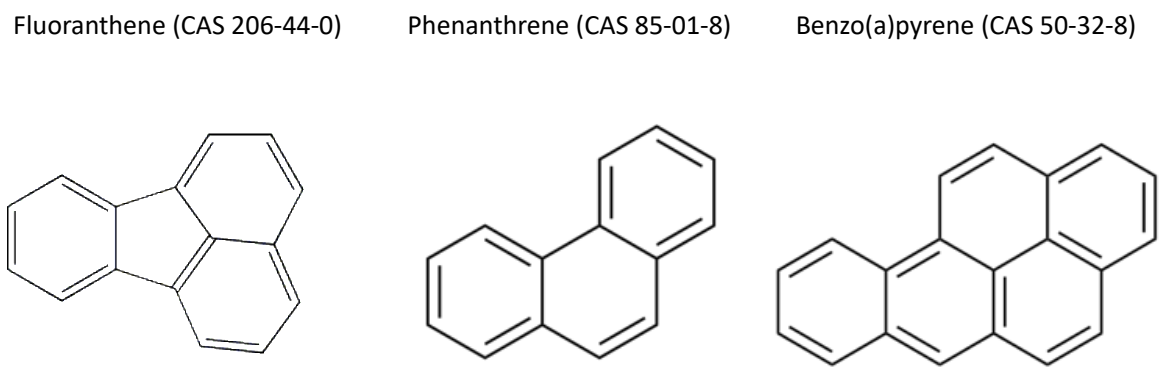

Figure 5: Examples of PAHs prevalent in Wallonia raw water[76].

On the other hand, the wide use of non-persistent organic pesticides results in human exposure. Currently used and banned pesticides and herbicides and their metabolites can be found in water sources. Chlorpyrifos, an organophosphate pesticide, is currently used in the US and has been recently banned in the EU. Its application is in agriculture and households for control of a number of pests, including insects and worms. It inhibits acetyl cholinesterase in the nervous system, resulting in respiratory, myocardial and neuromuscular transmission impairment, leading to acute effects. However, it can be mutagenic, carcinogenic, cytotoxic, genotoxic, teratogenic, and immunotoxic for humans[77,78].

Common herbicides including chloroacetanilides (metolachlor), triazines (atrazine, chloridazon), and metazachlor have been detected in water samples (Figure 6)[76,79–81].

Chloridazon is authorized for use on sugar and fodder beets that might be fed to livestock. In rats, mice and dogs, it can affect body weight and displays liver and kidney toxicity, and it affects the gastric mucosa in dogs at very high dose after short-time administration[82]. Chloridazon and its main metabolite (desphenyl chloridazon) are stable after pasteurisation, baking/brewing/boiling and sterilization[83], and have been found to be the most abundant contaminants in Wallonia raw water (frequency of detection = 82% in 100 samples, C_{max} = 21500 ng/L)[76]. Metolachlor is used extensively in the US for the control of annual grass weeds, broadleaf weeds, and yellow nutsedge, and is also commonly used in corn, soybean, and peanut cultivation. It is more persistent in the soil than the other acetanilide herbicides and has been detected in groundwater ranging from 0.08 to 4.5 µg/L in the US[84] and also in the EU[85]. Moreover, it has been detected in edible fish species, threatening human and wildlife health[86,87]. Atrazine is used to prevent broadleaf weeds in crops such as maize and sugarcane. Exposure to atrazine is associated with fetal developmental defecits[88] or carcinogenic effects[89]. While the three above herbicides have been banned in the EU, the other herbicide metazachlor is still currently in use. It is a cell division inhibitor widely used for controlling broad-leaved weeds and annual grasses[85]. Chronic consumer exposure scenarios to metazachlor have shown that the metazachlor intake is well below the established toxicological reference values in EU[90].

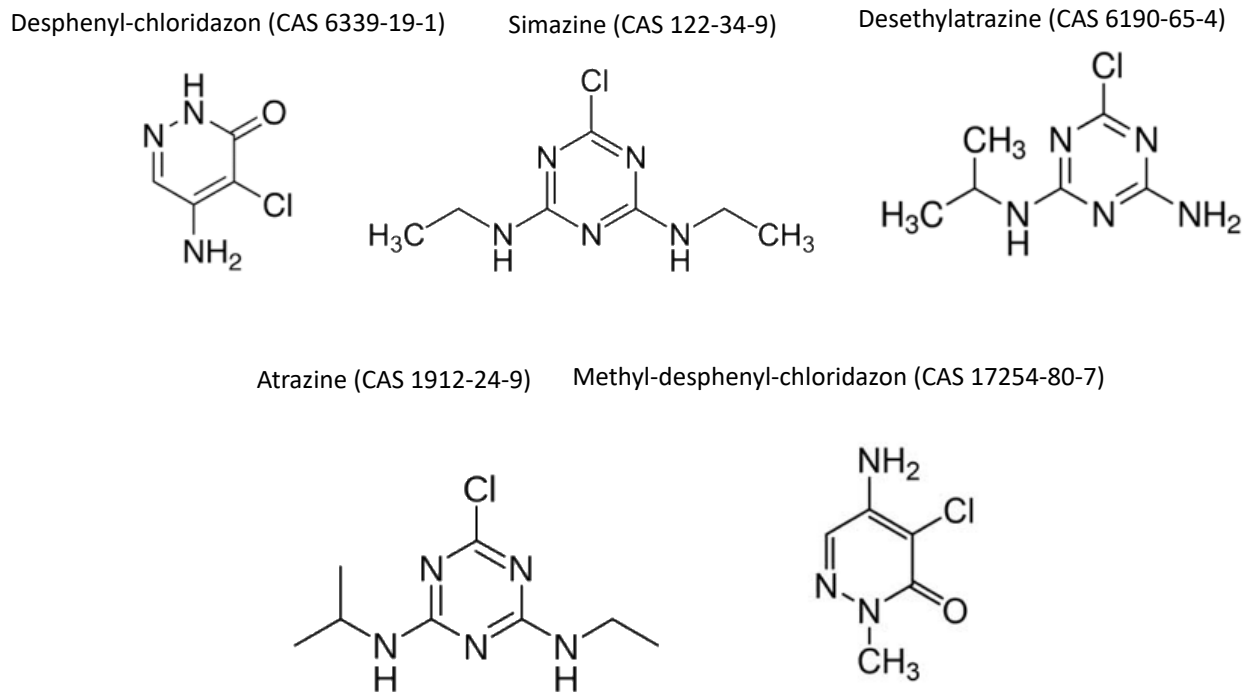

Figure 6: Examples of triazines, organochlorides and organophosphates prevalent in Wallonia raw water[76].

Bisphenol A (BPA) (Figure 7) is an extensively used chemical in the production of epoxy resins, polycarbonate plastics, and flame retardants[91]. It is one of the most commonly produced chemicals in the world with an industrial production volume of approximately 2.9 billion kilograms per year[90], therefore it became a worldwide contaminant[92]. Humans from all age groups are exposed to BPA mainly via food consumption[93], but other sources of exposure such as of air/dust and water also exist[90]. BPA has adverse effects on female and male reproductive systems in humans and animals[94], and it interferes with the development of the fetus and young children by interacting with estrogen, androgen and thyroid receptors[90], among other effects[38].

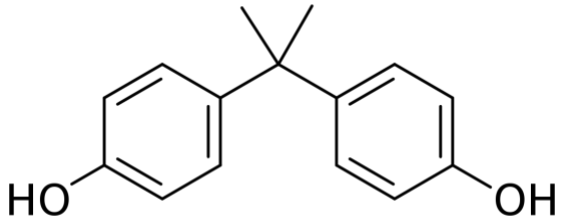

Figure 7: Structure of Bisphenol A.

1.3. Natural active compounds: polyphenols

Diets provide the major source of natural active compounds such as polyphenols[95–100]. Polyphenols are secondary metabolites of plants with a common polyphenol structure (*i.e.* several hydroxyl groups on aromatic rings). They are abundant in our diet originating from plants like fruits, vegetables, cereals, and coffee. They are naturally produced to aid host defenses against herbivores, insects, ultraviolet radiation, and microorganisms[101]. They include several thousands of molecules with a wide range of complex structures[102,103]. Based on the number of phenolic rings, polyphenols can be classified in many classes but mainly as phenolic acids, flavonoids, stilbenes, phenolic alcohols and lignans (Figure 8)[102].

The chemical structures of polyphenols determine their biological properties and bioavailability[102]. Most of the polyphenols display low oral bioavailability due to gastro-intestinal absorption and extensive metabolism. Polyphenols are popular in research and food manufacture since they have significant antioxidant properties and potential roles in preventing various diseases associated with oxidative stress such as cancer, cardiovascular, and neurodegenerative diseases[104]. Also, they are responsible for the activities of many medicinal plants by modulating the activity of a wide range of enzymes and receptors[105].

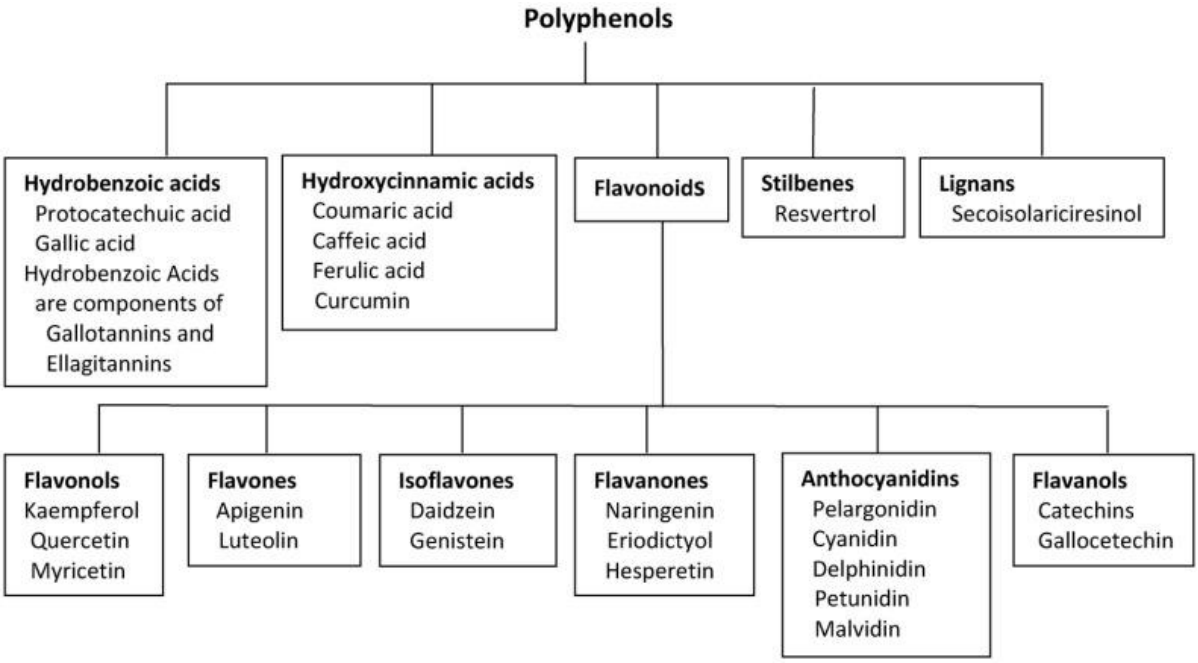

Figure 8: Polyphenol classifications[104].

Phenolic acids include two classes namely hydroxycinnamic and hydroxybenzoic acids, derivatives of cinnamic acid and benzoic acid, respectively. They are found in free forms in vegetables and fruits and in bound forms as β-glycosides (*i.e*. bound to one or more sugar molecules) in hull, bran, and seed. Curcumin is a well-known member of the family of hydroxycinnamic acids. It is extracted from turmeric, a spice derived from the rhizomes of the plant *Curcuma longa Linn*. Turmeric has been used in Asia for medicinal purposes for centuries due to its anti-inflammatory and anticancer activities[106].

Curcumin is poorly bioavailable primarily due to poor absorption, rapid metabolism and elimination, leading to its extremely low serum levels[107,108]. Therefore, instead of curcumin itself, the metabolites are detected in plasma or serum following oral consumption[109]. Curcumin concentration was shown to peak at 0.41 to 1.75 μM after one hour of oral intake of 4 to 8 g of curcumin in a clinical trial conducted in Taiwan[110]. Curcumin is metabolized mainly in liver[111]. Curcuminoids, as shown by clinical trials, are safe even at doses between 4000 and 8000 mg/day[112]. Curcumin is well known as a strong antioxidant by scavenging free radicals or modulating the enzymatic activity of antioxidant pathways, and also as an anti-inflammation agent in most diseases by blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[113].

Flavonoids include two benzene rings connected with three carbon chains from the nearby pyran ring with more than 5000 identified compounds. According to the variation in the type of

heterocycle involved, they are further classified into six classes: flavanones, flavanols, flavonols, isoflavones, flavones, and anthocyanidins (Figure 9). The estimation of the overall concentration of all flavones and isoflavones in circulation in human bodies is in the micromolar range[114]. Most flavonoids in edible plants and foods are in bound forms as β-glycosides[115]. Even after food processing and cooking, most flavonoid glycosides can reach the small intestine intact. While flavonoid aglycones (not bound to a sugar molecule) and a few flavonoid glycosides (bound to glucose) can be easily absorbed in the small intestine[116], glycosylated flavonoids have to penetrate into the mucus layer of the intestine to be deglycosylated on the cell surface prior to absorption. Otherwise, they may be hydrolyzed by bacterial enzymes in the colon[117], thus limiting their absorption in the colon[118]. Flavonoids are likely to appear as metabolites (*e.g.* of the phase II metabolites) in the bloodstream and urine due to rapid and extensive catabolism by the intestinal and liver cells[119].

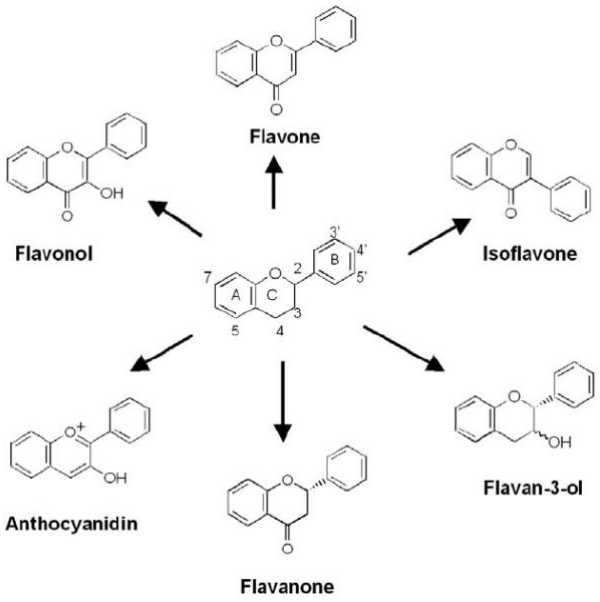

Figure 9: Basic structure of flavonoids[120].

The class of flavonols is the major class of flavonoids available in different food sources including onions, broccoli, apples, berries, teas, and leeks. Production of flavonols is activated by light, thus the flavonol content varies due to the light exposure of different parts of the plant [103]. Quercetin is a flavonol found frequently in onions, apples, broccoli, and berries[121,122]. Its daily intake by an US adult is 50 to 800 mg[123]. It is poorly soluble in water, but quite soluble in lipids. Quercetin has relatively low bioavailability due to its low absorption, extensive metabolism and/or rapid elimination (3% to 17% in healthy individuals receiving 100 mg)[122]. Quercetin is similar to curcumin, well known as a long lasting anti-inflammatory and strong anti-oxidant[121,124].

Flavones (*e.g*. chrysin and baicalin) are relatively less frequent flavonoids, found abundantly in fruit peel or skin (*e.g.* mandarin) and parsley, thyme, celery, and hot peppers. Chrysin is found in various medicinal plants including, for example, blue passion flower (*Passiflora caerulea*), but also in propolis and honey. It is poorly absorbed in the intestine. It can reach maximum concentrations of 12 to 64 nM in human serum within an hour after oral 400 mg doses of chrysin[114]. Chrysin suppresses the inducible nitric oxide synthase (iNOS) and NF-kB[125], and activates estrogen receptor[126]. It also has several other pharmacological activities[127]. Baicalin is also a flavone isolated from the root of *Scutellaria baicalensis* Georgi (Huang Qin). Due to baicalin's poor solubility in water, it is moderately absorbed in the stomach and poorly absorbed in the small intestine and colons, leading to an absolute bioavailability after oral administration of only 2.2%[128]. Baicalin plays a role in various pharmacological activities, especially preventing several cancers[129].

Isoflavones (*e.g.* daidzein and genistein) share structural similarities with estrogens with the presence of an –OH group in between C4 and C7 (Figures 9 and 10). They are able to bind to the estrogen receptor. Therefore, regular consumption of soybean-based products were showed to be associated with breast cancers and other deseases[130,131]. Their main source is soybeans, with dried soybean seeds containing approximately 1 g/kg of both daidzein and genistein[132]. The absolute bioavailability is 9–14% for genistein and 29–34% for daidzein in aglycone forms, but it has been shown to be close to 90% after oral administration of soy protein isolate in female Balb/c mice both using an administration of 1.2 mg/kg genistein and 0.55 mg/kg daidzein[133]. Barnes *et al.,* (1995)[134] calculated that the level of genistein in the blood of a person consuming 35 g/day of soybeans (the average amount consumed by the Taiwanese) can reach 3.3 pM, corresponding to an intake of 50 pg (185 pM) genistein with total body water (56 liters). Because both genistein and daidzein are relatively hydrophobic, they will be taken up by cells, therefore their distribution in the blood and tissues are relatively similar[134].

Resveratrol is a polyphenolic stilbene, naturally found in red grapes, red wine, peanuts, and groundnuts[135]. These plants produce resveratrol in response to stress, injury, fungal infection, or ultraviolet (UV) radiation[136]. Resveratrol usually occurs in *trans* and *cis* molecular configurations as glucosides[137]. Resveratrol appears to be well absorbed by humans when taken orally, but as the previous polyphenols, it has a poor bioavailability due to its rapid metabolism[138]. The blood concentration of resveratrol can peak at about 1.8 to 2 μM after one hour of a single administration of an oral dose of 25 mg of *trans*-resveratrol to healthy volunteers, depending on whether resveratrol is administered in wine, vegetable juice, or grape juice[137]. Resveratrol in red wine was initially thought to be responsible for red wine's

beneficial cardiovascular effects (the so-called "French Paradox"[137]), however, there is no strong evidence[139]. Resveratrol is recorded as an antioxidant and anti-inflammatory compound[140]. It also displays an estrogenic activity as it is able to compete with the estrogenic diethylstilbestrol for binding to the estrogen receptor, due to its similar chemical structure[140]. Figure 10 displays the structure of the seven polyphenols selected in this work, based on their common commercial availability as food supplements.

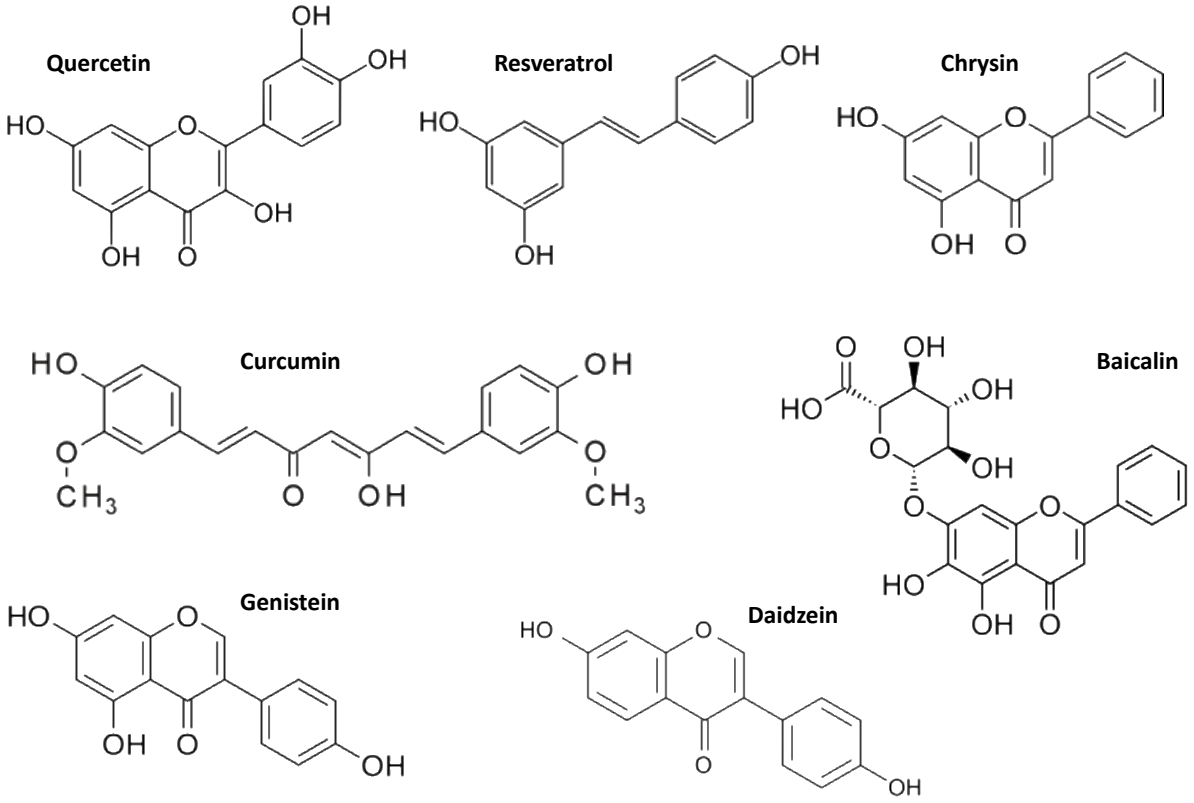

Figure 10: Structures of seven polyphenols.

2. Aryl hydrocarbon receptor and steroid receptors: Targets of these chemicals

Toxic chemicals can cause several health and environmental hazards, especially carcinogenesis and endocrine disruption. Endocrine disrupting activity is one of the main mode of actions and the most concerning adverse effect of these toxic compounds for the health of the current and future generations and the environment[141]. They are so-called endocrine disruptors (EDs). In 2002, the World Health Organization (WHO) defined EDs as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"[142,143].

Since our endocrine system requires a the hormone-coordinated harmonious development and functions of several organs and responds to very low hormone concentrations (part per

trillion to per billion range), exposure to EDs during vulnerable periods (development and reproduction) can induce adverse effects in the short and long terms, even at very low doses[141]. These compounds may interfere or interact with several target transcription factors that regulate gene expression including the master xenobiotic receptor, aryl hydrocarbon receptor (AhR) and the steroid receptors (glucocorticoid, androgen, estrogen, and progestogen receptors)[141,144].

2.1. Aryl hydrocarbon receptor

2.1.1. Evolution and function of aryl hydrocarbon receptor

Poland *et al*., (1976)[145] discovered the intracellular protein responsible for the aryl hydrocarbon hydroxylase activity in mouse liver treated with dioxin (TCDD). This protein appeared to be a receptor able to bind TCDD with an extremely high affinity. Therefore, it was called the dioxin receptor or the aryl hydrocarbon receptor (AhR).

AhR is a member of the basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) superfamily of transcription factors. Ligand-activated PAS proteins control circadian rhythm and regulate responses to environmental changes including both primary intracellular and secondary physico-chemical stimuli such as gas molecules, redox potential or photons[146,147]. Meanwhile, bHLH transcription factors in animal kingdom are involved in numerous developmental programs including cell migration and adhesion, DNA damage control and protection from oxidative stress, environmental adaptation, organogenesis, hematopoiesis, myogenesis, neurogenesis and sex determination[148]. In plants, they control growth, development, and stress adaptation by participating in various signal-sensing processes including detecting the signals of diatomic O_2, small metabolites *e.g*. phytoalexins and light[149].

AhR has been highly conserved throughout evolution[150]. Its homologs are present in almost all major groups of animals including the bilaterians, cnidarians and placozoans. Their last common ancestor, living approximately 600 million years ago, already possessed an AhR homolog[151]. AhR orthologs in *C. elegans*[152] and *D. melanogaster*[153] have no ligand binding capacity. Instead, they rather contribute to the neural development. Thus, it is likely that AhR evolved from a factor required for normal development and homeostasis regarding origin and evolutionary points of view, while the ligand activation may be the secondary and acquired function arising during vertebrate evolution[154,155].

Early researches on AhR mainly focused on understanding the molecular basis of toxicity induced by the typical AhR ligand, the dioxin TCDD[156]. Recent studies have shown that AhR

is a vital regulator of many essential biological processes such as immune responses, stem cell regulation, inflammation, cell differentiation and proliferation, apoptosis, reproduction, and tumor suppression, acting in response to endogenous ligands from diet, host cells or microbiota[157]. The toxicity resulting from exposure to dioxins or other xenobiotics is the consequence of the disruption of these processes. Therefore, AhR is considered as a sensor for environmental chemicals, which connects external environmental signals to cellular processes[158].

2.1.2. Structure of the aryl hydrocarbon receptor

The AhR structure consists of a N-terminal bHLH domain, a central PAS domain, and a C-terminal variable functional domain (Figure 11). The N-terminal bHLH domain functions as the DNA-binding domain and supports dimerization of the receptor with its their partners, for example the AhR translocation proteins (ARNT)[159]. The amino-acid sequence of this domain is extremely variable among species[160–162]. The bHLH domain helps AhR to bind to the cognate DNA sequences (5′-T(N)GCGTG-3′) called dioxin responsive elements (DREs) in the promotors of AhR regulated genes[163]. In this domain, AhR has a nuclear localization signal (NLS) and a nuclear export signal (NES) for nucleocytoplasmic shuttling.

The C-terminal segment of the AhR contains the transcriptional activation domain. This sequence contains three subdomains enriched with either acidic residues (glutamate/aspartate), or with glutamine (Q-rich) or with serine, threonine and proline (S/T/P). This segment is the interaction site for co-activators and co-repressors of AhR[164,165].

The PAS domain of the AhR has two imperfect repeats, PAS A and PAS B associated with DNA recognition, ligand binding and chaperone interactions. This domain, along with the N bHLH region, also supports the heterodimerization of the AhR with the ARNT[166,167]. The PAS B region of the AhR contains a ligand binding domain (LBD) and a binding site for heat shock protein 90 (Hsp90) (Figure 11)[168]. AhR is essentially expressed ubiquitously in mammalian tissues at various levels across tissues with liver, thymus, lung, kidney, spleen, and placenta being the tissues where AhR is the most expressed[169].

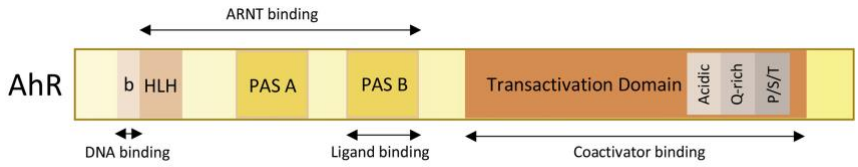

Figure 11: The functional domains of the AhR proteins.

2.1.3. The aryl hydrocarbon receptor signaling pathway

The inactive AhR forms a complex in the cytosol with a co-chaperone phosphoprotein p23, an AhR-interacting protein (AIP) also known as hepatitis B Virus X-associated protein 2 (XAP-molecule 2) and two Hsp90 proteins[170] (Figure 12). The AhR chaperone complex helps to stabilize the AhR in the cytosol and maintains its correct folding, thereby allowing the receptor to recognize the ligand properly, and subsequently ensuring the transcriptional activity of the AhR[171]. Several exogenous and endogenous AhR ligands from the environment, diet, host metabolism and gut microbiome can activate the AhR and induce its conformational alteration. Ligand-activated AhR translocates from the cytosol into the nucleus (Figure 12).

Two signaling pathways are possible, the canonical and the noncanonical (Figure 12). In the canonical signaling pathway, AhR dimerizes with ARNT to form an active heterodimer complex. The AhR/ARNT complex binds to the DREs in the promotors of AhR regulated genes[163], such as CYP1A (*CYP1A1, CYP1A2, CYP1B1*) or *AHRR* coding for a repressor (AhR repressor). That triggers the recruitment of various components of the transcriptional machinery to activate transcription[172]. Later, the AhR is exported back to the cytosol, followed by proteasomal degradation[173].

According to the noncanonical AhR pathway, regulation of the expression of genes lacking DREs could be explained by a direct AhR-DNA binding (without a binding site) or to a site distinct from the consensus DREs, the non-consensus DREs (*e.g*. 5′-GGGA-3′), or importantly, via a crosstalk with other signaling pathways (estrogen receptor (ER), NF-κB and retinoblastoma (RB)) (Figure 13)[174]. AhR can also function as an E3 ubiquitin ligase for proteasomal degradation, for instance, of ERα[175].

The best-studied AhR-responsive genes are detoxification enzymes for drug and xenobiotic metabolism[70]. They play an essential role in the overall responses of animals to toxic chemicals. Their activity is easy to measure, particularly in the case of *CYPs* such as *CYP1A1*, due to their low basal expression and tremendously rising expression induced by many dioxin-like chemicals. Thus, assessments of the actions of dl-chemicals based on this approach have provided very unambiguous and well-definable markers for AhR specific actions[176]. Indeed, methods measuring the expression of *CYP1A1* are widely accepted for determining AhR transcriptional activation[177]. Therefore, risk assessments in this field of science and the worldwide regulatory guidelines have been developed based primarily on the classical mode of action of the ligand-activated AhR[176].

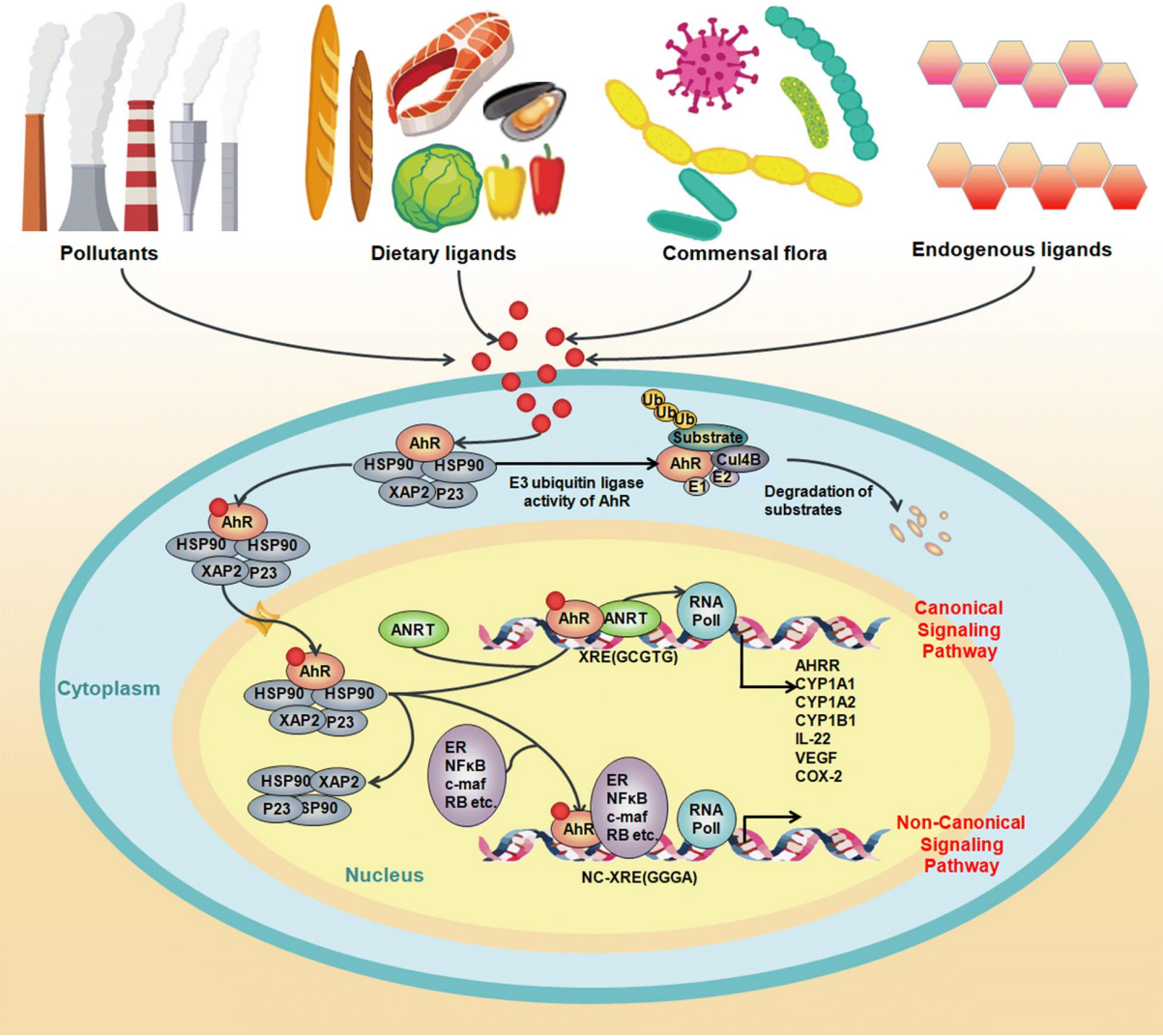

Figure 12: AhR modes of action in mammalian cells and the putative mechanism of AhR activation as described in the text[178].

2.1.4. The aryl hydrocarbon receptor ligands

Although the AhR is initially linked to toxic halogenated aromatic compounds (*e.g.* PAHs, dioxins, POPs), more recent studies show that the AhR interacts with more structurally diverse ligands[179,180]. AhR ligands can be endogenous or exogenous (natural or synthetic) compounds (Figure 13). According to their spatiotemporal availability, AhR ligands act differentially or similarly, depending on the context resulting in species-specific, ligand-binding specific and ligand-specific AhR functionality[181,182].

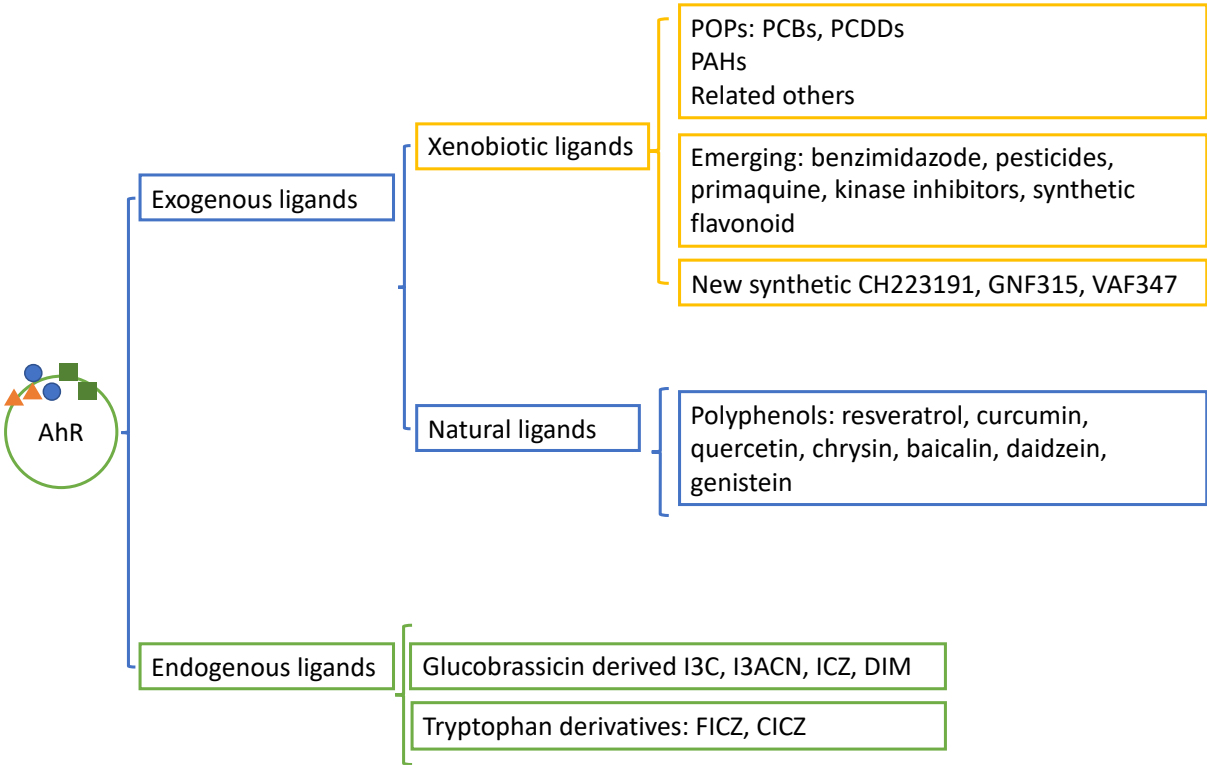

Figure 13: Exogenous and endogenous ligands of AhR[183].

The essential amino acid tryptophan (Trp) is the precursor for the synthesis of AhR ligands. Its catabolism either by the host or by the microbiota yields numerous physiologically relevant AhR ligands. The first observation was the production of endogenous AhR ligands causing AhR activity *in vitro* and *in vivo* after exposure to ultra-violet radiation[184,185]. Later, some photooxidized Trp derivatives were discovered to be endogenous ligands of AhR, suggesting a role for AhR in the skin[186]. FICZ, the product of irradiation of Trp solutions by ultraviolet and visible light[187–189], is considered as the highest affinity AhR ligand up to date[190,191] with an estimated K_d of 0.07 nM (TCDD K_d = 0.48 nM). In addition, the kynurenine pathway (KP) accounting for > 90% of the degradation of the dietary Trp, which transforms Trp into nicotinamide adenine dinucleotide and different intermediate byproducts, has been revealed as the major physiological route from Trp to produce AhR ligands[192]. These Trp derivatives are potential AhR ligands, while kynurenine has been identified as an AhR ligand precursor, which requires further chemical conversions to act as a AhR agonist[192] (Figure 14).

Studies of xenobiotic agonists like the dioxins (*e.g.* TCDD) and PAHs (*e.g.* benzo[*a*]pyrene; BaP) show that AhR prefers elongated planar compounds with large lateral extensions and small medial extensions along with specific medial H-bond potential[194,195]. However, thousands of xenobiotic compounds and cellular metabolites with diverse shapes and chemical properties have been reported to bind AhR[180,196]. A majority of them have an overall

elongated planar shape and some barely have any AhR ligand structural signature. This suggests that a flexible extended loop of AhR, named the “belt,” is longer and more flexible than other PAS family transcription factors, allowing the unique ability of AhR to interact with diverse ligands[192] (Figure 15).

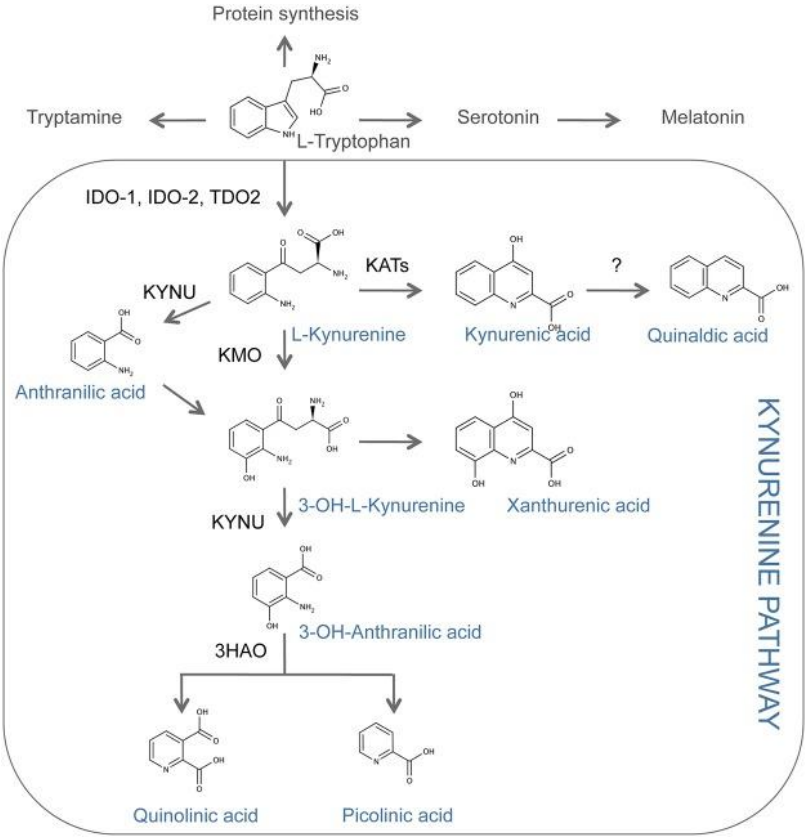

Figure 14: Kynurenine pathway of tryptophan degradation. KATs: kynurenine aminotransferases; KYNU: kynureninase; KMO: kynurenine 3-monooxygenase, IDO1/2: 2,3-dioxygenase; TDO1/2: tryptophan 2,3,-di- oxygenase; 3HAO: 3-hydroxyanthranilate oxygenase[193].

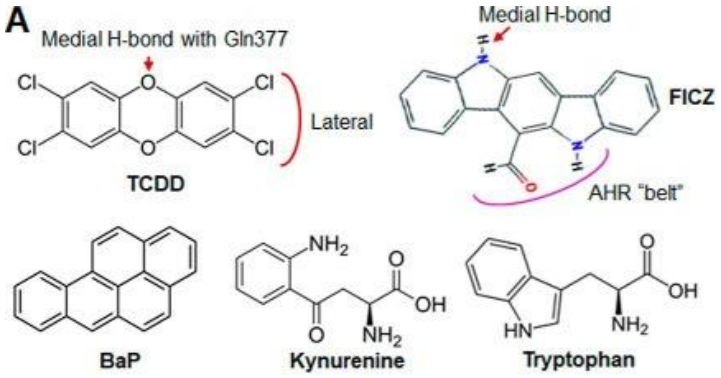

Figure 15**:** Chemical structures of well-known AhR ligands: TCDD, FICZ, BaP, and kynurenine, compared with tryptophan. TCDD and FICZ have chemical signatures of medial H-bond potential, facing lateral extension and the belt of the AhR[192].

On the other hand, polyphenols have various biological functions in addition to antioxidant properties which are yet poorly understood[103], especially their interaction with the AhR. Botanicals have been previously shown to affect AhR activation, and the major active compounds were polyphenols. For example, a concentration of 10 µM resveratrol has been shown to induce a 6-fold AhR activation in human mammary epithelial cells, MCF-10A cells[197]. Coffee, as a source of polyphenols, has been shown to inhibit BaP-induced production of oxidative stress via the activity of AhR[198]. Polyphenol extracts of strobiles of *Humulus lupulus*, *e.g*. *L., Cannabaceae* are able to upregulate CYP1A1 via the AhR mediated up-regulation of CYP1A1[199,200].

2.2. Steroid receptors

2.2.1. Evolution of steroid receptors

Nuclear receptors belong to a superfamily of transcriptional regulators which play key roles in animal development, physiology, and reproduction. Many nuclear receptors are activated by a specific ligand *e.g.* hormone or other small molecule, but also by postranslational modifications, and association with other proteins or DNA, depending on the cellular context[201].

Steroid receptors are members of the type I nuclear receptors, which are receptors for classical steroid hormones. Steroid hormones are small, lipophilic hormones synthesized from a common precursor molecule, cholesterol. They are produced by seemingly subtle modifications of the four fused rings of the sterol skeleton and side chains through a complex biosynthetic process in tissues and glands throughout the body. They activate the steroid receptors at levels as low as nanomolars[202]. Although mineralocorticoids, glucocorticoids, estrogens, progesterones, and androgens share structural similarities, they interact specifically with their own receptor to exert their biological effects (mineralocorticoid receptor (MR), glucocorticoid receptor (GR), estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR))[203] (Figure 16). From the discovery of ERα by Jensen at the end of the 1950's, through Gustafsson's description of the three-domain structure of GR in 1978, followed by the evidence that that the receptors act by binding to DNA at steroid hormone responsive elements (HREs), to the identification of a large family of nuclear receptors, comprising many "orphan receptors" without a known ligand; the study of nuclear receptors has been pioneering in many aspects of molecular Biology research, as summarized by Gustafsson (2016)[204].

Steroid receptors regulate the transcriptional activation of their downstream genes by recruiting co-regulators/co-activators to the pre-initiation machinery. They regulate genes which control a wide variety of biological processes such as cell proliferation, development, metabolism, and reproduction. They display well-described functions in the reproductive tract and endocrine system, but also play distinct physiologic roles[205]. Although ERα, ERβ, PR, and AR are crucial for sexual differentiation and reproduction via their hormone ligands, they regulate fundamental cellular functions such as growth, metabolism and homeostasis in both sexes[206]. Meanwhile, GR primarily regulates general physiology such as maintaining general homeostasis, whereas MR controls electrolyte balance and fluid transport.

2.2.2. Structure of steroid receptors

The structure of steroid receptors, typical for the nuclear receptors, contains five distinct domains including a variable amino-terminal region (A/B), a highly conserved DNA-binding domain (DBD) (C), a highly variable hinge region (D), a moderately conserved ligand-binding domain (LBD) (E), and an extremely variable C-terminal domains (F) (Figure 16). DBD and LBD contain specific crucial amino acid residues which determine the dimerization and function of the receptors. Finally, there are also one or two regions called activation function 1 and 2 (AF1 and AF2) for transactivating gene expression. AF1 located in the A/B domain is usually ligand-independent, whereas AF2 in the LBD is predominantly ligand-dependent[205].

Hydrophobic ligand binding pockets (LBPs) within the LBDs are composed of roughly 30 amino acids, and intended for binding lipophilic steroid hormones. The amino acids sequence is highly specific for binding to particular classes of steroid hormones so that minor changes can lead to dramatic changes in the hormone specific activation of the steroid receptors[207]. For example, a conserved cysteine residue in the human PR, GR, and MR appears to be critical for interacting with the C20 keto group in progesterons, glucocorticoids, and mineralocorticoids. A substitution of a threonine in the AR by a cysteine allows the receptor to have a high affinity for progesterone and corticoids and decreases its affinity for androgens[208].

The DBD is very highly conserved, showing 60–95% homology among the steroid receptors[209]. The function of the DBD is to bind to specific hormone response elements (HREs) in the promoters of their unique sets of target genes. The DBD contains two Cys_4 zinc fingers: the first finger (C1) interacts with the HREs, while the second (C2) participates in receptor dimerization and stabilizes DNA binding[210]. HREs have a palindromic (symmetrical) sequence with two “half-sites” from which each half is in contact with a monomer in the homodimer complex.

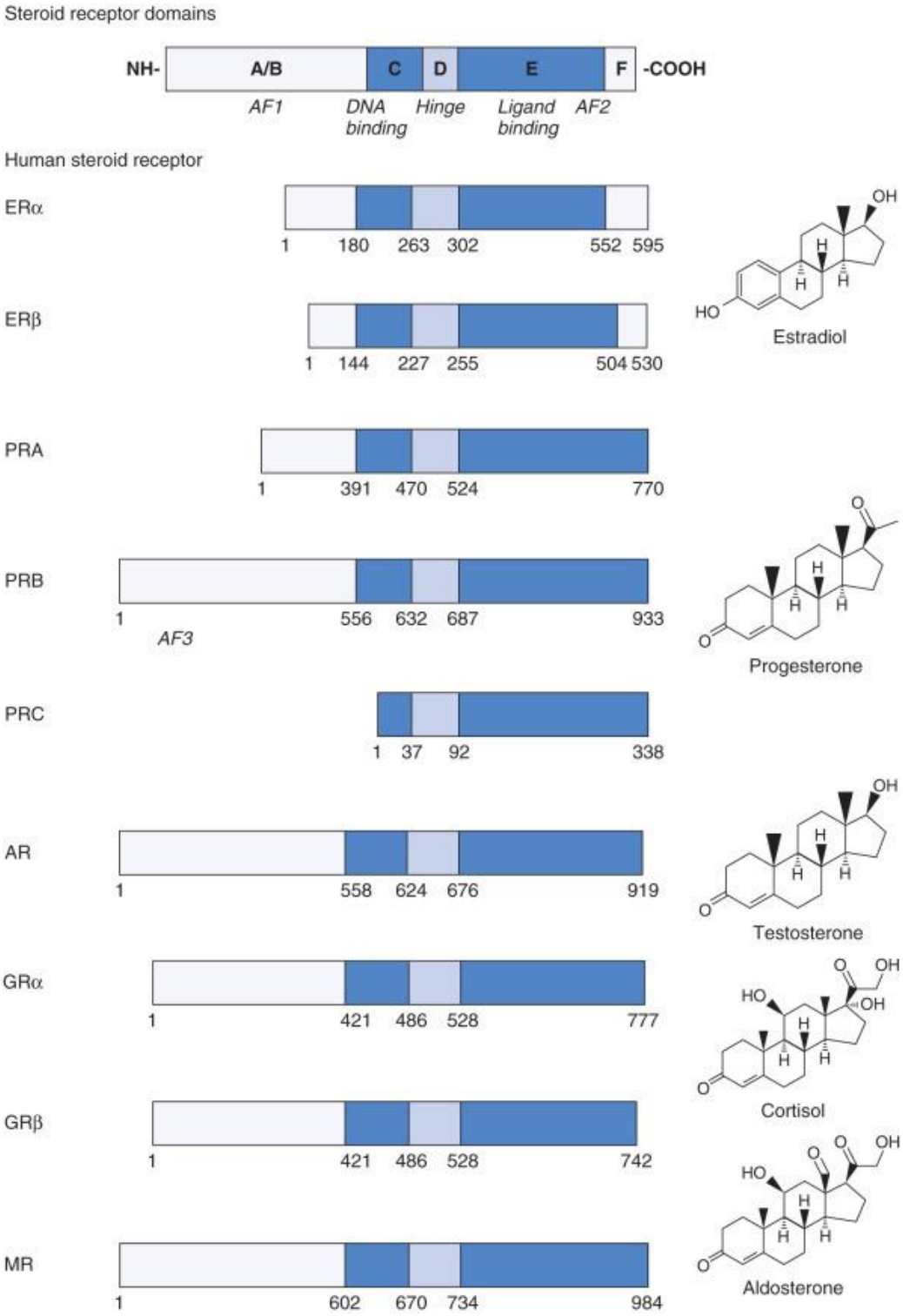

Figure 16: Primary structures of a generic steroid receptor and its functional domains, their isoforms, and their physiological ligands. Region A/B contains transactivation function 1 domain (AF1). (C): containing the DNA-binding domain (DBD); (D): the various hinge region; (E): containing the ligand-binding domain (LBD); (F): containing the transactivation function 2 (AF2) domain. AR: Androgen receptor; ER: estrogen receptor; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; PRA: progestin receptor isoform A; PRB: progestin receptor isoform B[205].

2.2.3. The steroid receptor signaling pathway

The canonical signaling pathway of steroid receptors also involves the binding to DNA consensus sequences, the HREs. The overall distribution of steroid receptors in specific cell compartments remains controversial[211]. The early model assumed that unbound steroid receptors are in the cytoplasm, bound to a chaperone complex (hsp90 and hsp70). The chaperone complex maintains the proper folding of the receptors in inactive conformation and facilitates hormone binding. The binding of the hormones to the ligand binding pocket (LBP) triggers conformational changes of the receptors and dissociation of the chaperones, resulting in the exposure of the nuclear localization domain, resulting in the dissociation of the chaperones. After that, the ligand-activated steroid receptors are translocated into the nucleus to bind their HREs and stimulate or inhibit transcription of their target genes (Figure 17). Most of the steroid receptors (such as ERs) have to form homodimers to be transcriptionally active (Figure 18)[212,213]. The homodimerization of the receptors rely crucially on LBD and DBD during ligand inducible conformational change[205].

While in the absence of ligand, GR and MR have been reported to reside in the cytoplasm in association with the chaperone hsp90, hsp70, and other receptor-associated proteins[211,214], ERα[214], ERβ[215], PR[216], and AR[216] and the nuclear receptor type 2 have been reported to be located in the nucleus, bound to DNA in the absence of hormones (Figure 17). The more recent model suggests that these receptors are inactive while they are bound to a complex of corepressor proteins[217,218] to keep chromatin in a more condensed conformation by association with proteins functioning as histone deacetylase.

While ER binds to a specific estrogen response element (ERE), 5′-GGTCAnnnTGACC-3′GR[216], the four other receptors AR, PR, MR, and GR share the same HRE called the glucocorticoid response element (GRE), 5′-GGTACAnnnTGTTCT-3′ (with n = any nucleotide)[219], due to their highly conserved DBD. Although these four receptors share a common high-affinity consensus DNA-binding site *in vitro*[220], differential specific binding to HREs may be caused by the discrimination through nucleotides in the spacer and flanking regions of the half-sites and by asymmetries in the half sites[220].

Differences in the DNA binding sequence affect GR conformation and regulatory activity[221]. Such an allosteric regulation has also been seen for ERs with an effect on the recruitment of coactivators[222,223]. The distance between the HREs and the promoter of the target gene has been shown to affect the transcriptional activity of steroid receptors[224]. There is also a tissue- or gene-selective effect of the steroid receptors depending on their modulators, especially in case of compounds modulating the activities of GRs and ERs[225]. Some selective ER

modulators (SERMs) can induce estrogenic activity in some tissues, but anti-estrogenic activity in other tissues. For example, tamoxifen is an ER agonist in the bone and uterus, whereas it is an antagonist in breast and endometrium[225]. The reason lies in the variable tissue-specific coregulator pools or the variable conformation of ER upon SERM binding, which determines the specific recruitment of coactivators versus corepressors by ERs[226].

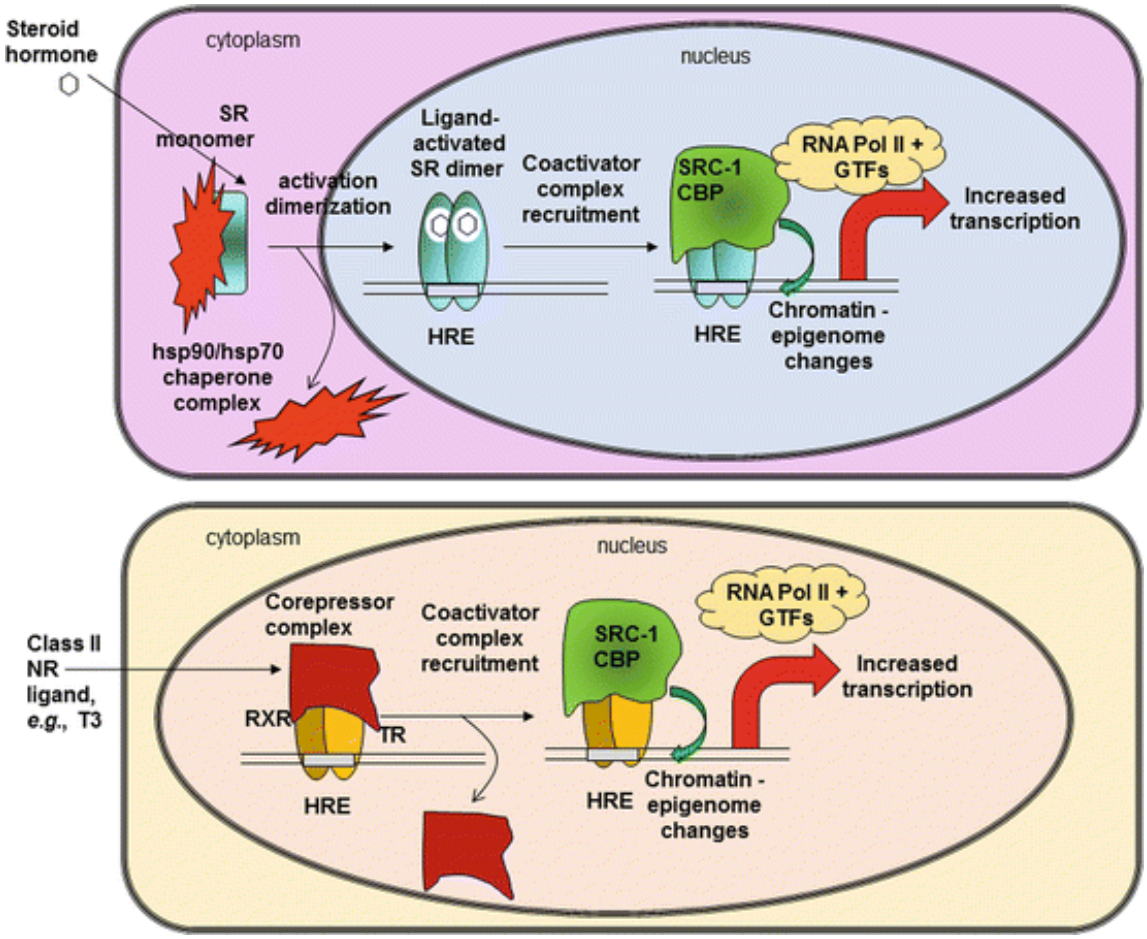

Figure 17: General mechanism of action for cytoplasmic steroid receptors as described in the text. SR: Steroid receptors; HRE: hormone response element[211].

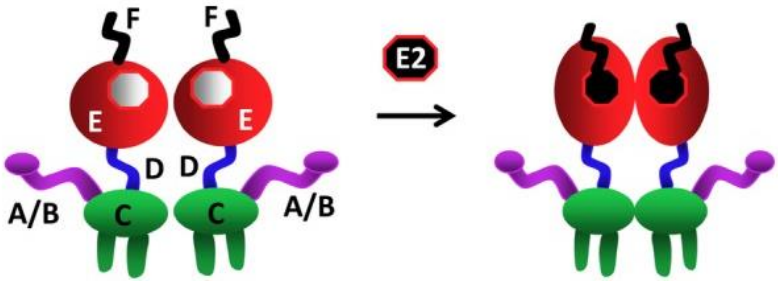

Figure 18: The ERs are dimers with or without the endogenous ligand, 17β-estradiol (E2), the binding of which induces conformational changes in the receptors[212,213].

2.2.4. The steroid receptor ligands

The steroid receptors are activated by their endogenous ligands such as mineralocorticoids, glucocorticoids, estrogens, progesterone, and androgens from which they take their name from (mineralocorticoid receptor (MR), glucocorticoid receptor (GR), estrogen receptor (ER), progestin receptor (PR), and androgen receptor (AR)). However, a wide range of synthetic chemicals can affect the activity of the steroid receptor signaling pathways. For example, benzo(a)pyrene (BaP) is known as a potent endocrine and growth disruptor, able to alter multiple endocrine and growth factor pathways[227], whereas bisphenol A is an estrogenic endocrine disrupting compound, shown to activate the ER-mediated pathways[228–230]. Indeed, the steroid receptors are primary targets of EDs. EDs have been shown to bind to the steroid receptors with affinities ranging from sub-nanomolar to high micromolar in several biochemical and cell-based assays with some mechanisms being revealed by structural analyses[144].

3. Cell-based Chemical Activated LUciferase gene eXpression (CALUX) assays

One of the modes of action of EDs is to bind and alter the activity of the steroid receptors[231] which could be extended to AhR considering its versatile role of AhR in development and homeostasis. To evaluate the health effects of these chemicals, the transcriptional activity of the receptors induced by the chemicals can be investigated. Transactivation gene expression assays, also called reporter gene assays, have been introduced as less expensive, more high-throughput-amenable, and more routinely applicable screening technics. They have been internationally validated to provide mechanistic and semi-quantitative data (by Environmental Protection Agency (EPA) of the US as well as by the Organization for Economic Cooperation and Development (OECD))[232,233]. The mechanistic data from the *in vitro* assays about the interaction between the receptors and chemicals could serve as the first-line screening method for narrowing down further in-depth bioanalytical investigations on the chemicals. Indeed, a good correlation has been reported between *in vitro* and *in vivo* results, when available[234,235].

The Dioxin Responsive Chemical Activated LUciferase gene eXpression DR-CALUX assays measure the transcriptional activity of the AhR through the expression of a luciferase reporter gene under the control of a recombinant promotor containing DREs. It is one of the most commonly used assays for screening toxicity of compounds via the canonical AhR signaling pathway (Figure 19). The induction of gene expression resulting from the activation of the AhR signaling pathway responding to chemicals is the basis of this bioassay system[236]. The recombinant cells are stably transfected with an AhR-inducible firefly luciferase reporter gene which carries several DREs. The DREs can be synthetic[237] or from native AhR-regulated genes such as the *CYP1A1* promotor[238] to respond to dioxins and any other chemical(s) that

can bind to and activate the AhR. Induction of the luciferase gene expression is directly dependent on the presence of transcriptionally active AhR; it will reflect the appearance of activated AhR in a time-, dose-, and chemical-dependent manner, also reporting the potency of the tested chemicals[239]. The DR-CALUX assay has been devoted for detecting the AhR ligands (mainly dioxins and dl-compounds) in food and feed[240,241], as well as in human and wildlife samples[242–244]. It can detect dioxins and dl-PCBs in feed at part per trillion (ppt) levels, which is comparable to High Resolution Gas Chromatography Mass Spectrometry (HR-GC/MS)[12]. Similarly, the steroid receptor reporter cell lines are also based on their canonical signaling pathway involving the binding to the HREs and activation of the transcription of its target genes[245–247].

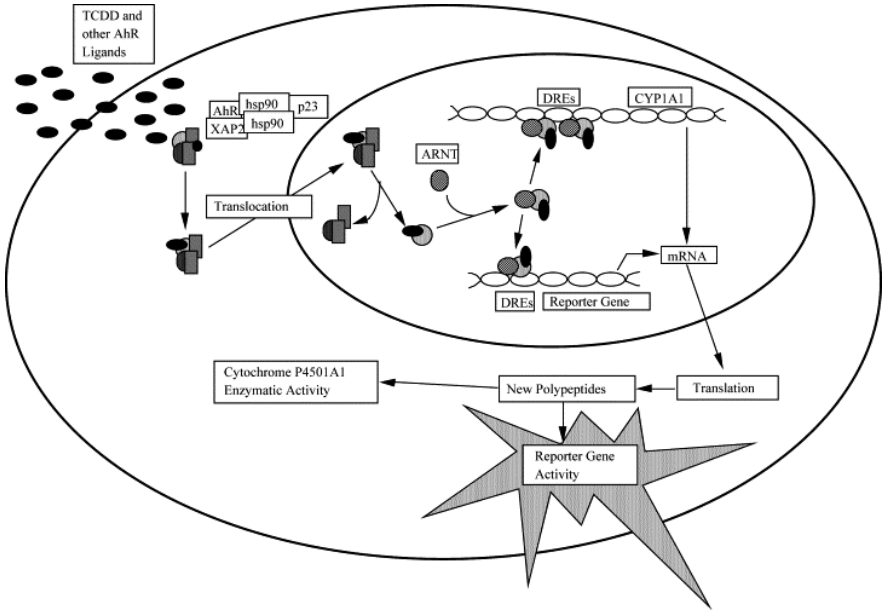

Figure 19: Molecular mechanism of induction of gene expression by TCDD and related AhR agonists as described in the text[239].

The reporter gene assays offer some advantages over the other currently used *in vitro* assays which are also based on the canonical signaling pathway of the steroid receptors. For example, the reporter gene assays reproduce accurately the signaling pathway of a compound (*e.g*. via DREs or HREs controlling gene expression), while the comparable gene expression assays show directly the transcription of the genes without evaluating further the transcriptional pathway[248]. The reporter gene assays are able to discriminate between agonistic and antagonistic activity (by the co-exposure with a typical agonist) while the comparable receptor-binding assays did not[245]. Moreover, the reporter gene assays are "untargeted" and able to reveal the complex response of mixture effects[245,249,250].

4. Mixture effects of chemicals

While, the present chemical risk assessment mainly relies on a chemical-by-chemical approach[251], understanding mixture toxicity is crucial to assess the potential adverse effects resulting from a real life exposure to mixtures of chemicals[252]. Mixture toxicity is the adverse effect of mixtures of chemicals after simultaneous or sequential exposure, also named as *combination effect*, *combined effect* or *joint action*[253]. The effects can be additive, synergistic, or antagonistic depending on whether they are equal, stronger, or weaker than the sum of the effects of individual components, respectively[254]. Kortenkamp *et al*., (2009)[253] introduced the concept called "something from nothing" and "a lot from a little" to describe the mixture effect. This concept suggests that a mixture containing several compounds, each at a concentration at which they have no effect individually, can have an effect.

Generally, mixture studies can be approached by “whole mixture” or “component based” methods. The former assesses the toxicity for a given mixture of chemicals as a whole, considering its activity as the activity of this specific mixture. The approach seems to be cost effective and feasible for many types of mixtures. However, there are many possible mixtures and the mixtures themselves can change their compositions and doses[255]. Therefore, a component-based approach, which estimates the mixture toxicity based on the studying of its components, gains favor in evaluating mixture toxicology. This approach allows to focus on modes of action and prediction of the mixture effects based on the activity of each components, and also allows to reveal the most active compound(s) in the mixtures.

4.1. Predictions and assessments of the combined toxicity of mixtures of chemicals based on the component approach

Due to the countless combinations of doses and components that one mixture can be composed of, mathematical models have been applied to establish the quantitative relationship between the toxicities of single compounds and that of their mixtures. Hence, the mixture effect can be predicted by using the toxicity data of the individual compounds. The non-interaction or additivity assumes that the chemicals in the mixture act together but do not interact *i.e.* inhibiting or enhancing the toxicity of the others. This assumption becomes the default for predicting and assessing the mixture effect. Dose concentration addition (CA) and independent action (IA) are described by the additivity expectation. The deviations from the expected additive effects could be synergisms or antagonisms, but their magnitude cannot be formulated. Without evidence of synergisms or antagonisms, additivity will be applied.

Concentration addition (CA). CA introduced by Loewe and Muischneck (1926)[256], has been applied for the chemicals with the same mode of actions (MOAs). The CA model assumes that all the chemicals behave as if they are simply the dilution of one another. CA has been successfully applied for mixtures of chemicals with an identical, well-defined molecular target such as mixtures of organophosphorus pesticides, photo-synthesis-inhibiting herbicides and polychlorinated dioxins and furans, and also estrogenic agents *etc.*, however, it has also proven powerful for mixtures with dissimilar compounds[257–259].

The predicted concentration to obtain a specific effect by the mixture (EC_{mix}) can be calculated by taking into account the concentration partition (p_i) of compound i and its respective activity (EC_i)[256,257,260]. CA is also known as "Toxic unit summation"[261], in which "toxic unit" (TU_i) is the ratio of the actual concentration/dose (c_i) of a substance in the mixture to cuase an equivalent effect (EC_i) of the substance if present alone (Eq. 1). Toxic units reflect the effect of a given concentration of a substance to its toxicity and the additivity assumption means that they can be totally or partially replaced by the other without changing the overall effect of the mixture. Therefore, the mixture effect (EC_{mix}) (the following Eqs. 2, 3, 4, and 5) is simply the sum of the toxic units, and the contribution of individual chemicals to the mixture effect is in proportion to its toxic unit (*i.e.* its concentration and potency)[262].

$$(1) TU_i = \sum \frac{c_i}{EC_i}$$

$$(2) TU_{mix} = \sum TU_i$$

or

$$(3) \frac{c_{mix}}{EC_{mix}} = \sum \frac{c_i}{EC_i}$$

or

$$(4) EC_{mix} = \frac{C_{mix}}{\sum \frac{c_i}{EC_i}} = \frac{\sum c_i}{\sum \frac{c_i}{EC_i}}$$

or

$$(5) EC_{mix} = \left(\sum \frac{p_i}{EC_i} \right)^{-1}$$

where both EC_{mix}, the concentration of the mixture and EC_i, the concentration of the chemical i, exert the same specific effect, p_i is the fraction of chemical i in the mixture, c_{mix} is the total mixture concentration.

The "Toxic Equivalence Factor" (TEF) for the assessment of PCDD/F[250] is one example of using CA model to predict the mixture effect from the effect of the individual components. In DR-CALUX assays, the doses of all PCDD/F congeners can be expressed in terms of the

dose of TCDD (the reference compound) needed to generate the same equivalent effect. Therefore, the toxicity of the mixture can be predicted simply by adding up all equivalent dose of TCDD. Besides TEF, several other CA derived methods have been suggested for mixture risk assessment such as the Hazard Index (HI), Point of Departure Index (PODI), Relative Potency Factors (RPF) and Generalized concentration addition (GCA). Hazard Index[263] can be expressed as the ratio between the exposure level and the reference/acceptable level. This method can use flexible uncertainty factors when defining reference levels. Point of Departure Index[264], on the other hand, is also based on the fraction of exposure levels but relative to their respective points of departure (PODs), which could be No Observed Adverse Effect Levels (NOAELs), or No Observed Effect Concentrations (NOECs) or Benchmark doses (BMDLs). By that, it removes different uncertainty factors from the calculation. GCA assumes that the hillslope for each component is equal to 1 and considers also their maximum activities, therefore GCA allows to generate the full dose-response curve of the mixture using only the data from testing the individual compounds[265].

Independent action (IA). The model, first applied for biological data by Bliss (1939)[266], is based on a probabilistic evaluation of independent random events with the joint probability $\rho_S(c_i, c_{i+1})$ for the survival/tolerance of an individual exposed to a combined concentration $c_{mix} = c_i + c_{i+1}$:

$$(7)\rho^S(c_i, c_{i+1}) = \rho^S(c_{mix}) = \rho^S(c_i) * \rho^S(c_{i+1})$$

The probability of the deadth of the individual is $\rho_D = 1 - \rho_S$, thus, the probability of dying of the individual when co-exposed to substance i and i+1 is:

$$(8)\rho^S(c_{mix}) = 1 - \rho^S(c_i, c_{i+1}) = 1 - [\rho^S(c_i) * \rho^S(c_{i+1})] = 1 - [(1 - \rho^D(c_i)) * (1 - \rho^D(c_{i+1}))]$$

From that, the resulting combined effect can be calculated from the effects of each component and was successful in some applications[260,262]. At a specific concentration, E_{mix} is the effect of the mixture; E_i and E_{i+1} are the effects of chemicals i and i+1 at that specific concentration.

$$(9)E_{mix} = 1 - ((1 - E_i)(1 - E_{i+1}))$$

The IA model (Eq. 9) considers that replacing one component by another may or may not be equivalent. The mixture effect at a specific concentration can be predicted from the dose response curve of each constituent applied independently[266]. Since IA is based on probabilistic background, it assumes strictly the range of 0 to 1 (0% to 100%) for an Euclidean-type effect parameter and a monotonic effect (entirely non-increasing or entirely non-decreasing) concentration-response curves for the individual mixture components[262]. It is applied to

estimate the combined effect of dissimilar compounds, assuming that chemicals act completely independently and have no influence on each other.

4.2. Grouping criteria

Grouping chemicals with similar toxicity is equally important in evaluating the toxicity of the mixture. Chemicals with similar MOAs in both nature and major biochemical events have been grouped according to "Toxicologic similarity", as proposed by U.S. EPA (2000)[267] and other bodies. It listed the criteria for grouping chemicals within the same MOAs with extensive guidance, for example pesticides. A recent approach for chemical toxicity classification has been introduced based on common adverse outcomes. This approach acknowledges the occurrence of a similar toxicological effect of chemicals despite different toxic mechanisms (*i.e.* metabolism, distribution and elimination) and broadens the grouping criterion based on MOAs.

In human toxicology, MOAs have been applied widely, so that chemicals acting via common mechanisms are often grouped together. The common mechanism of toxicity is considered when similarities exist in both nature occurrence and sequence of major biochemical events. However, it is not clear how precise MOA should be when classifying chemicals into a common group sharing the same MOAs, either based on their chemical structures or mechanistic subsequences. Therefore, grouping based on their MOA can be unrealistically narrowed and exclude chemical-induced effects. Presently, grouping criteria are established by focusing on common adverse outcomes. Although chemicals can act through different mechanisms, with different molecular details, biological effects can be similar, sharing a common endpoint or adverse outcomes. The approach of common adverse outcomes broadens the grouping scope, moving the focus to effect instead of mechanism.

Scopes and objectives

This PhD work was performed in the frame of the PROTECTED project funded by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 722634. "PROTECTion against Endocrine Disruptors; Detection, mixtures, health effects, risk assessment and communication".

The PROTECTED project aims to develop expertise and protective capabilities for developing novel tools/concepts and training for the detection, analysis and improved risk assessment of endocrine disruptors (EDs), especially in mixture forms. The project focuses on several methodologies including multiplexed analysis, mixture modelling, mechanistic and exposure studies, explants and cell or whole organism bioassays. The PhD candidate is the early stage researcher (ESR) number 9 among 15 ESRs. The work specifically addresses the work packages 7 for Biological Analytical Tools – "*In vitro* bioassays for the ED and toxicity risk assessment of EDs and their mixtures".

Chemicals are indispensable in human daily life. We are exposed to mixtures of chemicals through several routes mainly via food and water consumption. Therefore, understanding mixture toxicology is necessary for evaluating the risk of exposure to these mixtures of chemicals, especially EDs. It is also crucial to be able to identify the main culprit(s) of the mixture effect and to predict the mixture effect, thereby protecting the wellbeing of both humans and wildlife.

This research aims to study the impacts of exposure to chemicals in individual and in mixture forms on the transcriptional activity of the aryl hydrocarbon receptor (AhR) and the four steroid (estrogen, androgen, progesterone, and glucocorticoid) receptors, representing the targets for endocrine disruptors (EDs) using *in vitro* Chemical Activated LUciferase gene eXpression (CALUX) assays. Three mixtures covering several exposure routes of humans in daily basis, are being investigated:

(1) a mixture consisting of 29 POPs (persistent organic pollutants) prevalent in Scandinavian human blood

(2) a mixture of 18 potential EDs dominantly found in Wallonia raw water intended for drinking water production, and

(3) a mixture of seven dietary polyphenols selected on the basis of their commercial availability as food supplements.

Specific aims of the project are:

(a) evaluating species and tissue-specific AhR responses of the POP mixture and the polyphenol mixture and their components

(b) profiling the endocrine disrupting activities of EDs prevalent in raw water using AhR and steroid receptors

(c) identifying interactions among the chemicals (additive, antagonistic or synergic effects) on the AhR activity

(d) identifying the most active chemical(s) in the mixtures

(e) predicting the effect of the mixtures based on the activity of single compounds.

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Chapter 2

The Aryl hydrocarbon receptor (AhR) is involved in xenobiotic metabolism and controls many biological pathways. AhR functions can be dysregulated by inappropriate ligand activation or inhibition, leading to the subsequent health concerns. Many potential AhR ligands are persistent organic pollutants (POPs). They are persistent and widely distributed in the environment and in animal bodies, resulting in toxic health effects to both humans and wildlife. Humans are exposed to mixtures of POPs; however, their risk assessment is usually based on a chemical-by-chemical approach. Therefore, to assess the health effects associated with mixed exposures, knowledge on mixture toxicity and how to predict mixture effects are required. There are three mathematical models developed for predicting the mixture effects: i) the concentration addition (CA) model designed for chemicals with similar mode of actions; ii) independent action (IA) applied for chemicals with different mode of actions; and iii) the generalized concentration addition (GCA), a CA modified model developed for predicting the effects of mixtures containing partial agonists.

The chapter 2 assessed the AhR transcriptional activity of a defined mixture of 29 POPs (the total POP mixture) constructed based on their presence and concentration in blood of a Scandinavian population. Both AhR agonistic and antagonistic activities of 29 POPs individually and in mixtures were evaluated by using three transgenic cell lines (rat hepatoma DR-H4IIE, human hepatoma DR-HepG2 and human mammary gland carcinoma DR-T47-D). The aims were to (a) assess both AhR agonistic and antagonistic activities after exposing the cell lines to the 29 POPs and to the mixtures, (b) identify the main compound(s) responsible for the observed effects of the mixtures, and (c) predict the mixture activity by applying available mathematical models (IA, CA and GCA).

The results obtained were published in Environmental Pollution (Doan et al., 2019). **Doan**, T.Q.; Berntsen, H.; Zimmer, K.; Verhaegen, S.; Ropstad, E.; Connolly, L.; Igout, A.; Muller, M.; Scippo, M. A. Realistic Mixture of Persistent Organic Pollutants (POPs) Inhibits the Transactivation Activity of the Aryl Hydrocarbon Receptor (AhR) in Vitro. Environ. Pollut. 2019, 254, 113098. https://doi.org/10.1016/j.envpol.2019.113098

A mixture of persistent organic pollutants relevant for human exposure inhibits the transactivation activity of the aryl hydrocarbon receptor *in vitro*

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Abstract

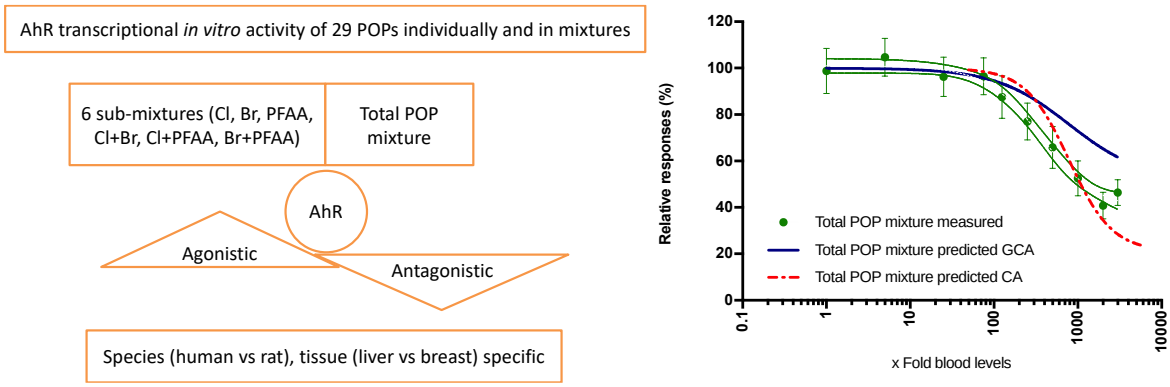
While humans are exposed to mixtures of persistent organic pollutants (POPs), their risk assessment is usually based on a chemical-by-chemical approach. To assess the health effects associated with mixed exposures, knowledge on mixture toxicity is required. Several POPs are potential ligands of the Aryl hydrocarbon receptor (AhR), which involves in xenobiotic metabolism and controls many biological pathways. This study assesses AhR agonistic and antagonistic activities of 29 POPs individually and in mixtures by using Chemical-Activated LUciferase gene eXpression bioassays with 3 transgenic cell lines (rat hepatoma DR-H4IIE, human hepatoma DR-Hep G2 and human mammary gland carcinoma DR-T47-D). Among the 29 POPs, which were selected based on their abundance in Scandinavian human blood, only 4 exerted AhR agonistic activities, while 16 were AhR antagonists in DR-H4IIE, 5 in DR-Hep G2 and 7 in DR-T47-D when tested individually. The total POP mixture revealed to be AhR antagonistic. It antagonized EC_{50} TCDD inducing AhR transactivation at a concentration of 125 and 250 and 500 fold blood levels in DR-H4IIE, DR-T47-D and DR-Hep G2, respectively, although each compound was present at these concentrations lower than their LOEC values. Such values could occur in real-life in food

contamination incidents or in exposed populations. In DR-H4IIE, the antagonism of the total POP mixture was due to chlorinated compounds and, in particular, to PCB-118 and PCB-138 which caused 90% of the antagonistic activity in the POP mixture. The 16 active AhR antagonists acted additively. Their mixed effect was predicted successfully by concentration addition or generalized concentration addition models, rather than independent action, with only two-fold IC_{50} underestimation. We also attained good predictions for the full dose-response curve of the antagonistic activity of the total POP mixture.

Keywords: Persistent Organic Pollutants; Aryl hydrocarbon Receptor; Antagonistic activity; Human relevant mixture; Generalized concentration addition model

Capsule: A mixture of persistent organic pollutants relevant to human exposure antagonized the transcriptional activity of the Aryl hydrocarbon receptor *in vitro*, whose activity could be predicted.

Graph abstract:



Highlights:

- Mixed exposure is more realistic for human exposure to POPs.
- Total POP mixture relevant for human exposure is AhR antagonistic.
- Chlorinated compounds are the drivers of the activity of the total POP mixture.
- PCB-118 and PCB-138 contribute for 90% of the total POP mixture effect.
- Dose-response curve of the mixture was predicted successfully by CA and GCA models.

1. Introduction

The aryl hydrocarbon receptor (AhR) was originally characterized as a xenobiotic mediator[1]. It is often called the “dioxin receptor” as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and several dioxin-like (dl) compounds are AhR agonists. Adverse health effects associated with exposure to these AhR agonists are widely studied, including abnormal reproduction and development, impaired immune system, liver toxicity and cancers[2].

AhR physiological roles have recently gained more attention since AhR is activated by a wide range of structurally diverse endogenous and exogenous compounds[3]. Also, AhR-deficient rodents suffer from various physiological defects in the immune system[4], liver[5], kidney[4,6], cardio-vascular system[7], urinary bladder[8], *etc*. Additionally, AhR homologs are preserved in animal evolution for 600 million years[9], while invertebrate AhR homologues cannot bind dioxin[10], indicating that dioxin detection was not the primary role of this protein. Increasing evidence supports important roles of AhR in normal development and homeostasis, while toxicity induced by AhR xenobiotic ligands could be due to perturbation of these normal processes[11].

Upon ligand binding, the AhR is translocated from cytosol into the nucleus where it joins its dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT). This AhR/ARNT complex then binds to a DNA sequence called dioxin responsive element (DRE) to activate the expression of a battery of genes, including both phase I and phase II xenobiotic metabolism enzymes, of which *cyp1a1* is the best characterized. Hence, methods measuring *cyp1a1* gene expression are widely accepted for determining AhR activation[12], among which cell-based screening methods such as Chemical-Activated LUciferase gene eXpression (CALUX) are the most common[13–15]. Many potential AhR ligands are persistent organic pollutants (POPs). POPs are resistant to degradation and widely distributed in the environment. They can be detected in almost every human sample, including fetuses and embryos[16]. They tend to bioaccumulate and biomagnify in living organisms, resulting in toxic health effects to both humans and wildlife[17].

Presently, chemical risk assessment mainly relies on a chemical-by-chemical approach[18]. In real life, humans are exposed not to an individual POP, but to highly complex POP mixtures[19]. Understanding mixture toxicity is crucial to assess the potential adverse health effects associated with such real life exposure to mixtures of POPs[20]. The mixture effects may be additive, synergistic, or antagonistic depending on whether they are equal, stronger, or weaker than the sum of the effects of individual components, respectively[21]. The concepts called "something from nothing" and "a lot from a little" were introduced to describe the mixture

effect[22] and proved in a study on fish when a significant effect was observed for a mixture combining individual compounds each at "no observed effect" concentrations[23].

Due to the various mixture forms and doses, models using the information of components to predict the combined effects are required. Three mathematical models have been developed for this purpose: i) the concentration addition (CA) model was designed for chemicals with similar mode of actions (MOAs)[24], but has also proven useful for mixtures with dissimilar compounds[23,25,26]; ii) independent action (IA)[27], successful in several applications[28,29], applies for chemicals which act independently and have different MOAs; and iii) the generalized concentration addition (GCA)[30], a CA modified model, was developed for predicting the effects of mixtures containing partial agonists[30–32].

In an effort to fill the gap in the knowledge of mixture toxicology, a defined mixture of 29 POPs (total POP mixture) was constructed based on their prominence in blood and/or food and breastmilk with the concentration being average blood values from recent Scandinavian studies[33–35] published prior to 2012[36]. To mimic the exposure of cells (in a tissue) to chemicals that are in the blood stream, in this study, the AhR transcriptional activities of the selected 29 POPs were determined individually and in mixtures (the total POP mixture and six sub-mixtures[36]) using reporter gene assays involving cancer cell lines from different tissues (liver and mammary gland) and species (rat and human). The aims were to (a) assess both AhR agonistic and antagonistic activities after exposing the cell lines to the 29 POPs and to the mixtures, (b) identify the main compound(s) responsible for the observed effects of the mixtures, and (c) predict the mixture activity by applying the three available models (IA, CA and GCA).

2. Materials and Methods

2.1. Chemicals and suppliers

The total POP and six sub-mixtures were designed and premade by the Norwegian University of Life Sciences, Oslo, Norway as described[36]. The former consists of 29 POPs, where most of them are listed as POPs under the Stockholm Convention on Persistent Organic Pollutants, belonging to three groups: six perfluorinated compounds (PFAAs), seven brominated compounds (BFRs), and 16 chlorinated compounds with seven polychlorinated biphenyls (PCBs) and nine organochlorine pesticides (OCPs). The latter consist of either one single class of compounds (PFAA, Br and Cl) or two combined classes (Cl+Br, Cl+PFAA, Br+PFAA). This way of mixture preparation was to enable the study of the effect of adding or removing one chemical group on different endpoints. As the design of the mixtures was focused on compounds occurring at high concentrations, most dl-PCBs (with the exception of PCB-118)

and polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/PCDF) were deliberately excluded. These compounds were also omitted due to their high toxicity at low concentrations in several systems to allow the study of the effect of the non-dl and most prevalent compounds. The components included in the mixtures and their respective concentrations are given in Table S1.

Along with the mixture testing, 29 POPs from the total POP mixture were also examined individually. They were bought from Sigma Aldrich (Missouri, USA) except *o*-chlordane from Toronto Research Chemicals (North York, Canada) and PCB-118 from Dr Ehrenstorfer (Augsburg, Germany). All chemicals were dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Molinons, France), except HCB in hexane (Merck, Massachusetts, USA).

The 29 individual POPs and the mixtures were stored as stock solutions at -20$_{o}$C. Working solutions were prepared from the stock solutions to reach the concentrations mentioned in Table S1. The highest tested concentration was 50 μM for all PCBs and PFAAs and 20 μM for BFRs except BDE-100, BDE-153, BDE-154 and BDE-209 (1 μM) due to stocks available. OCPs were tested at the maximum concentration of 100 μM for *γ*-HCH and dieldrin, or 80 μM for all the others. The concentrations for mixture exposure are presented as "fold blood levels", relative to the average contaminant levels found in human blood of the Scandinavian population. The total POP mixture and sub-mixtures were tested at concentrations between the estimated concentrations in human blood and maximum 3000 fold blood levels.

2.2. Determination of aryl hydrocarbon receptor agonistic and antagonistic activities

2.2.1. Cell-based assays

Rat and human dioxin responsive (DR) cell lines were used. Rat hepatoma DR-H4IIE cells were from BioDetection System (Amsterdam, The Netherlands) while both human cell lines (hepatoma DR-Hep G2 and mammary gland carcinoma DR-T47-D) were previously home-made (Liege, Belgium)[37]. A vector containing an AhR-controlled luciferase reporter gene was stably integrated into these cells. The vector integrated into DR-H4IIE cells contained four native DREs (from the upstream promotor of the mouse *cyp1a1* gene), leading the MMTV (Mouse Mammary Tumour Virus) promoter[38], while both DR-T47-D and DR-Hep G2 cells were transfected with a vector containing four synthetic DREs regulating the thymidine kinase promoter[37]. The cells were routinely cultured in MEMα (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% v/v fetal bovine serum (Greiner, Kremsmünster, Austria), 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma Aldrich, Missouri, USA), and incubated in a H_20 saturated atmosphere containing 5% CO_2, at 37°C.

Table S1: Composition of the total POP mixture and the six sub-mixtures (PFAA, Br, Cl, Cl+Br, Cl+PFAA, Br+PFAA). The minimum (Min) and maximum (Max) tested concentrations of each compound in individual compound testing and the concentrations of each compound corresponding to 1000 fold blood levels in the mixture testing are also given.

Compounds	**C Individual testing:**		**Mixture testing: individual concentrations in the mixture**						
	Min	Max	Total POP	PFAA	Br	Cl	Cl+Br	Cl+PF	Br+PFAA
PCB-28	0.01	50	0.031			0.031	0.031	0.031	
PCB-52	0.01	50	0.021			0.024	0.021	0.021	
PCB-101	0.01	50	0.025			0.025	0.021	0.025	
PCB-118	0.01	50	0.14			0.13	0.12	0.13	
PCB-138	0.01	50	0.43			0.45	0.39	0.42	
PCB-153	0.01	50	0.70			0.73	0.63	0.67	
PCB-180	0.01	50	0.34			0.38	0.33	0.35	
***p,p'*-DDE**	0.5	50	1.066			1.088	0.95	1.006	
HCB	0.075	60	0.23			0.24	0.21	0.22	
***α*-chlordane**	0.4	80	0.024			0.029	0.032	0.032	
***o*-chlordane**	0.4	40	0.34			0.034	0.029	0.029	
***t*-nonachlor**	0.5	50	0.099			0.099	0.101	0.101	
***α*-HCH**	0.5	50	0.017			0.017	0.017	0.017	
***β*-HCH**	0.5	50	0.076			0.076	0.069	0.069	
***γ*-HCH**	0.5	100	0.017			0.021	0.017	0.017	
Dieldrin	0.5	100	0.055			0.063	0.060	0.058	
BDE-47	0.025	20	0.019		0.012		0.016		0.014
BDE-99	0.025	20	0.0071		0.005		0.007		0.0071
BDE-100	0.0025	1	0.0035		0.003		0.004		0.0035
BDE-153	0.0025	1	0.0016		0.001		0.002		0.0016
BDE-154	0.0025	1	0.0031		0.003		0.003		0.0016
BDE-209	0.0025	1	0.0094		0.010		0.009		0.0094
HBCD	0.025	20	0.055		0.055		0.033		0.065
PFHxS	0.5	50	7.809	6.71				7.41	6.91
PFOS	0.5	50	41.52	18.19				40.83	16.37
PFOA	0.5	50	4.209	7.24				4.42	6.69
PFNA	0.5	50	1.092	1.76				1.036	1.74
PFDA	0.5	50	0.38	0.72				0.44	0.66
PFUnDA	0.5	50	0.34	0.32				0.17	0.28
Summary			59.048	34.94	0.091	3.43	3.101	57.51	32.75

POPs: persistent organic pollutants. PFAA: perfluorinated compounds. Br: brominated compounds. Cl: chlorinated compounds. PCB: polychlorinated biphenyl. DDE: dichlorodiphenyldichloroethane. HCB: hexachlorobenzene. HCH: hexachlorocyclohexane. BDE: brominated diphenyl ether. HBCD: hexabromocyclododecane. PFHxS: perfluorohexanesulfonic acid. PFOS: perfluorooctanesulfonic acid. PFOA: perfluorooctanoic acid. PFNA: perfluorononanoic acid. PFDA: perfluorodecanoic acid. PFUnDA: perfluoroundecanoic acid.

The methodology for the DR-CALUX (Dioxin Responsive Chemical-Activated LUciferase gene eXpression) bioassays was described in detail elsewhere[37,39]. Briefly, cells were first seeded in white clear-bottomed 96 well microplates (Greiner, Kremsmünster, Austria) and incubated for 24h to reach about 90% of confluence in the well. After 24-hour exposure, the cells were washed with phosphate buffered saline (Sigma Aldrich, Missouri, USA) and treated with lysis solution (containing Triton X100, Sigma Aldrich, Missouri, USA). Luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) were then added to the cell lysate to produce luminescence, which was measured by using a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). The cells were exposed, in triplicates, to a dilution series of the tested compound/mixture in both agonistic and antagonistic tests. For the latter, the cells were co-exposed with a constant concentration of 15 pM, 150 pM and 650 pM TCDD corresponding to TCDD EC_{50} in DR-H4IIE, DR-T47-D and DR-Hep G2 cells, respectively.

In order to verify whether AhR antagonists compete for the same, single site on the AhR with the agonist (TCDD), additional antagonistic tests were performed for selected compounds by co-exposing DR-H4IIE cells to different concentrations of the tested compounds and a constant saturating TCDD concentration (20 nM). Using the agonist (TCDD) at clearly saturating concentrations would make it impossible for a lower affinity antagonist to affect transcriptional activation at all.

All the exposure experiments were repeated at least three times independently. The final concentration of DMSO in the culture medium for the single POPs was 0.2% and 0.3% for agonistic and antagonistic tests, respectively, while they were 0.3% and 0.4% for the mixtures. For quality control, a TCDD reference curve was performed on each plate.

MTT cell viability and LDH cell cytotoxicity were performed along with visual inspection of cell morphology and attachment. The former was carried out in a replicate plate to the DR-CALUX assays. After 24-hour exposure, 25 μL MTT dye solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml, Sigma Aldrich, Missouri, USA) was added into each well, followed by a 4-hour incubation at 37°C to form insoluble purple formazan. Then, 100 μL isopropanol (Merck, Massachusetts, USA) was added into the plates to dissolve the formazan for two hours. The MTT formazan absorbance was read at 550/630 nm by a spectrophotometer (ELx800™ BioTek, Winooski, USA). Because the MTT data need to be interpreted with caution and is not necessarily related to cell death, we performed the LDH cell cytotoxicity as well. The Pierce™ LDH Cytotoxicity Assay Kit was purchased from Thermo

Fisher Scientific (Massachusetts, USA) and operated according to the manufacturer's instructions (absorbance at 490/630 nm).

2.2.2. Calculations of EC_{50}, IC_{50} and efficacy (RPC_{Max})

Final results were presented as relative responses, *i.e.* percentages of the cell response to the tested compound/mixture compared to the maximum response of the cells to TCDD on the same plate for agonistic activities, or to spike-in TCDD EC_{50} for antagonistic activities. Dose-response curves were generated by Graphpad PRISM software, version 7 (San Diego, California, USA) by fitting a four-parameter non-linear regression for agonistic (Eq. 1) or antagonistic (Eq. 2) tests.

$$(1)\ Y_{agonistic} = B + \frac{x^H(T - B)}{x^H + {EC_{50}}^H}$$

$$(2)\ Y_{antagonistic} = B + \frac{T - B}{1 + \frac{x^H}{{IC_{50}}^H}}$$

where x is the concentration of a tested compound/mixture inducing the relative response $Y_{agonistic}$ or $Y_{antagonistic}$. EC_{50} and IC_{50} are the half maximal effective concentration for an agonist and antagonist, respectively[40]. B = bottom, T = Top, H = Hillslope.

The lowest observed effect concentration (LOEC) is the lowest tested concentration at which a significant effect ($p < 0.05$) was observed. The maximum observed effect concentration (MOEC) is the lowest tested concentration causing the maximum effect ($p < 0.05$). ANOVA (Graphpad PRISM) was used to determine statistical significance. Prior to ANOVA, tests for homogeneity of variance and normal distribution (transformation if needed) were performed. When no full dose-response curve was achieved, MOEC was considered as the highest concentration of the test series. Efficacy was determined as RPC_{Max} (%), which is the maximum effect induced by the tested compound[40]: AhR agonistic RPC_{Max} was the maximum relative response of the compound/mixture compared to the maximum TCDD response, while AhR antagonistic RPC_{Max} was the minimum relative response observed by the maximum inhibition of the test compound/mixture to the spike-in TCDD EC_{50}. The compound/mixture was accepted as active when its relative response was higher than the threshold level $RPC_{Max} \geq 10\%$ for AhR agonists and lower than $RPC_{Max} \leq 70\%$ for AhR antagonists[40].

2.2.3. Calculations of the predicted mixture antagonistic effects

Concentration addition (CA). CA model is based on a dilution principle, all the chemicals behave as they are simply the dilution of one another in the mixture. Hence, the effect contribution of one compound to the mixture effect can be totally or partially replaced by the

effect of the other. It calculates the effect concentration ($IC_{mix,j}$) of the mixture inducing a specific antagonistic effect j (from 1% to 100%) by considering the concentration partition (p_i) of compound i and its respective effect concentration (IC_{ij}) inducing the same effect j (Eq. 3). Previously published formulae were adapted[24,25,29]:

$$(3)\ IC_{mix,j} = \left(\sum_{i=1}^{n} \frac{p_i}{IC_{ij}}\right)^{-1}$$

The concentration partition p_i can either consider or not the non-active (NA) compounds. Because nonactive compounds do not give IC_{ij}, n is the number of the active compounds.

For each compound i, IC_{ij} inducing the effect j is calculated using its $IC_{i,50}$ and hillslope (H_i) from their fitted curves using Eq. 4 (Graphpad PRISM):

$$(4)\ IC_{ij} = IC_{i,50}\left(\frac{j}{100-j}\right)^{1/H_i}$$

Because the CA model allows only the calculation of $IC_{mix,j}$, to generate the full dose-response curves, we proposed several possible methods to calculate the hillslope and bottom (H_{mix} and B_{mix}) of the mixture response, while the top was set to 100% as no response.

- the Weighted Mean Hillslope and Bottom (WMHB) (Eq. 5a) considering p_i to weight the hillslope and bottom of the individual compounds:

$$(5a)\ H_{mix} = \left(\sum_{i=1}^{n} p_i H_i\right)\frac{100}{n}\ or\ B_{mix} = \left(\sum_{i=1}^{n} p_i B_i\right)\frac{100}{n}$$

- the Averaged Hillslope and Bottom (AvBH) considering the average of the hillslope and bottom of the individual compounds (Eq. 5b):

$$(5b)\ H_{mix} = \frac{\sum_{i=1}^{n} H_i}{n}\ or\ B_{mix} = \frac{\sum_{i=1}^{n} B_i}{n}$$

- the Formulated Hillslope and Bottom (FoBH) (Eq. 5c) using the formulae of the CA:

$$(5c)\ H_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{H_i}\right)^{-1}\ or\ B_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{B_i}\right)^{-1}$$

Independent action (IA). This method assumes that the effect of each component is an independent event[27]. Thus, the probability to exert a specific effect of the mixture is the joint probability of the effect of each compound applied independently. For calculating the relative response of the mixture, the data for individual compounds were converted into a probability.

An antagonistic effect induced by compound i is obtained by subtracting the measured relative response (R_{ik}) from 100% (100% being the relative response of TCDD EC_{50}) and then converted into a probability (scale 0-1, by dividing by 100). The relative response of the mixture (0%-100%) is calculated from the combination of individual probabilities of each compound using the adapted formula (Eq. 6)[28,29]:

$$(6)\ R_{mix,k} = 1 - \left(1 - \prod_{i=1}^{n}\left(1 - \frac{100 - R_{ik}}{100}\right)\right)$$

At a specific concentration k, $R_{mix,k}$ is the relative response of the mixture; R_{ik} is the relative response of compound i at that concentration k of the mixture, n is the number of the active components.

Generalized concentration addition (GCA). GCA (Eq. 7) assumes that the hillslope for each component is equal to 1 and considers also their RPC_{Max31}. It was adapted for AhR antagonistic activity similarly to the IA model by assuming 100% as no effect:

$$(7)\ R_{mix,k} = 100 - \frac{\sum_{i=1}^{n}\frac{RPC_{Max,i}C_{ik}}{IC_{50i}}}{1 + \sum_{i=1}^{n}\frac{C_{ik}}{IC_{50i}}}$$

where $R_{mix,k}$ is the relative response of the mixture at a specific concentration k, C_{ik} is the concentration of compound i in the mixture at that specific mixture concentration k. $RPC_{Max,i}$ is the maximum effect of compound i and IC_{50i} is the IC_{50} of compound i.

3. Results

In DR-H4IIE cells, while α-chlordane caused cytotoxicity at the highest tested concentration of 80 μM, *t*-nonachlor already did at 62.5 μM in three cytotoxicity tests (data not shown). They were also cytotoxic for the DR-T47-D at lower concentrations of 30 and 30.5 μM, respectively. These cytotoxic concentrations were excluded from the data analyses. None of the other compounds or mixtures induced cytotoxicity at any tested concentration.

3.1. Aryl hydrocarbon receptor activities of the 29 POPs

AhR – mediated agonistic activities. Only four out of the 29 tested POPs presented AhR agonistic activities ($RPC_{Max} \geq 10\%$). BDE-153, PCB-138, and PCB-118 were active in DR-H4IIE, while BDE-99 was active in DR-T47-D. BDE-99 and BDE-154 were able to trigger a weak agonistic activity ($5\% < RPC_{Max} < 10\%$) in DR-H4IIE, as well as γ-HCH in DR-T47-D (Table S2). No agonistic response was recorded in DR-Hep G2 cells for any of the 29 POPs.

Table S2: EC_{50}, RPC_{Max} and relative potency for AhR agonistic compounds in DR-H4IIE and DR-T47-D cells (n = 3, 0.2% DMSO).

Cell lines	DR-H4IIE					DR-T47-D	
Compounds	BDE-99	BDE-153	BDE-154	PCB-118	PCB-138	BDE-99	γ-HCH
EC_{50} ± SE (μM)	4 ± 0.8	ND	ND	25 ± 13	28 ± 6.4	1.9 ± 1.8	> 50
RPC_{Max} (%)	7.3	15.2	8.9	61.3	28.2	11	6
Relative potency	0.0000038	-	-	0.00006	0.000054	0.000013	0.000033

EC_{50}: concentration inducing half of the maximum response, extrapolated; RPC_{Max}: observed efficiency of the maximum tested concentration expressed in % of the maximum response of TCDD; Relative potency: ratio between the EC_{50} of TCDD and the EC_{50} of the tested compound, with TCDD EC_{50} = 15 pM in DR-H4IIE and 150 pM in DR-T47D; SE: Standard Error; ND: not determined.

***AhR – mediated antagonistic activities*.** Sixteen out of the 29 individually tested POPs displayed AhR antagonisms. No antagonistic activities were observed for any of the PFAAs in all three cell lines. In contrast, in DR-H4IIE cells, AhR antagonistic responses were recorded for 16 POPs including all PCBs, most of the OCPs (except *p,p'*-DDE, α-HCH and β-HCH), and three out of the seven BRFs (BDE-47, BDE-99 and HCBD) (Table 1). PCB-118 and PCB-138 displayed a V-shaped dose-response curve, switching from antagonistic to agonist behavior at concentrations above 3.5 μM and 27.5 μM, respectively. Hence, their IC_{50} values were determined by only the antagonistic part of the curve. The dose-response curves obtained from DR-H4IIE cells co-exposed to TCDD EC_{50} and the 16 AhR antagonistic POPs are shown in Figure S1 (solid lines) with a detail in Table S3.

DR-Hep G2 cells were less responsive to the POPs than DR-H4IIE, with only five compounds exerting antagonistic activities, namely PCB-28, PCB-118, PCB-138, HCB and BDE-47 (Table 1). PCB-28 was the most potent compound, almost completely abolishing the activity of 650 pM TCDD in DR-Hep G2, displaying an RPC_{Max} of 7.2 ± 3.6% and an IC_{50} of 6.1 ± 1.4 μM. In DR-T47-D cells, seven out of the 29 POPs showed AhR antagonistic activities (PCB-28, PCB-118, PCB-138, HCB, α-chlordane, *t*-nonachlor and γ-HCH). The highest potencies were found for α-chlordane and *t*-nonachlor with RPC_{Max} of 7.4 ± 12.5% and 27.8 ± 3.5%, respectively.

Evaluating competitive inhibition of 16 antagonists in DR-H4IIE. The AhR antagonistic activities were abolished for all compounds (except α-chlordane and *t*-nonachlor with RPC_{Max} of 56.6% and 56.8%, respectively) when co-exposing with excessive 20 nM TCDD, indicating they are possible AhR competitive antagonists (Figure S1, dashed lines).

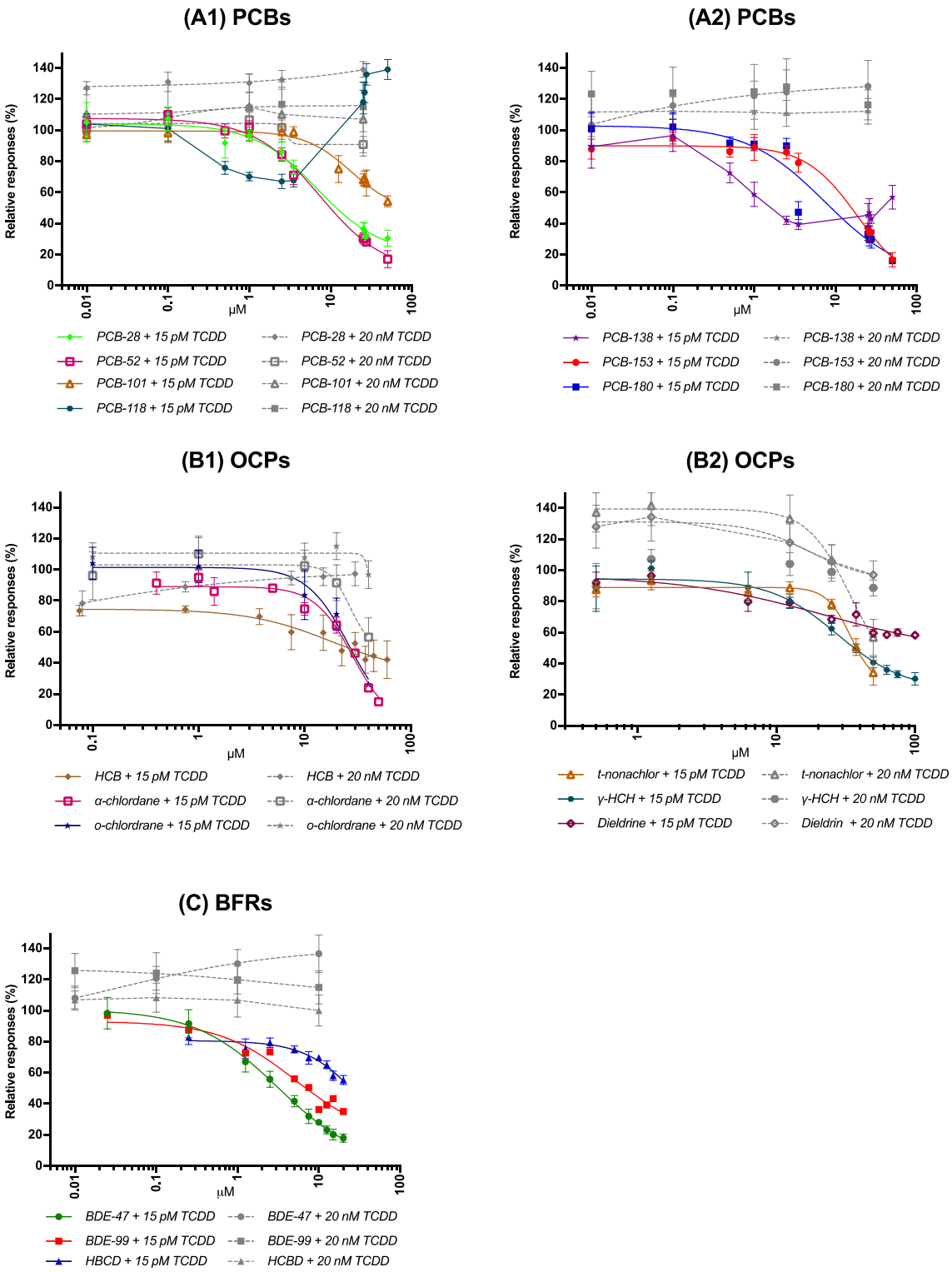


Figure S1: Dose-response curves obtained from DR-H4IIE cells co-exposed to 15 pM TCDD (solid lines) or 20 nM TCDD (dashed lines) and PCBs (A1 and A2), OCPs (B1 and B2) and BRFs (C) (Mean ± SD, n = 3, 0.3% DMSO).

Table 1. AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of 16 POPs in DR-H4IIE, DR-Hep G2 and DR-T47-D cell lines (n = 3, 0.3% DMSO).

Compounds	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)
PCB-28	2.5	25	6.8 ± 1.7	36.6 ± 4.3	2.5	25	6.1 ± 1.4	7.2 ± 3.6	3.5	25	11.4 ± 1.5	48.3 ± 5.9
PCB-52	2.5	50*	7.3 ± 1.2	17.1 ± 5.5	-	-	-	-	-	-	-	-
PCB-101	12.5	50*	17.9 ± 3.8	54.3 ± 3.6	-	-	-	-	-	-	-	-
PCB-118	0.5	2.5	0.3 ± 0.05	67 ± 4.7	12.5	25	9 ± 2.7	38.3 ± 16.5	12.5	27.5	13.6 ± 2.4	49.2 ± 4.9
PCB-138	0.5	2.5	0.6 ± 0.07	42.8 ± 2.8	3.5	25	ND	50.2 ± 9.5	12.5	50*	ND	42.6 ± 2.5
PCB-153	0.01	50*	18.5 ± 2.8	16.7 ± 4.6	-	-	-	-	-	-	-	-
PCB-180	1	50*	7.4 ± 3.3	16 ± 1.4	-	-	-	-	-	-	-	-
HCB	0.075	37.5	17.9 ± 11.6	42 ± 8.7	3.75	37.5	4.5 ± 2.3	39.4 ± 12.7	0.075	30	16.4 ± 2.1	51.7 ± 6.7
α-Chlordane	0.4	50*	28.3 ± 3.3	15 ± 1.1	-	-	-	-	10	30	20.2 ± 2.1	7.4 ± 12.5
o-chlordane	20	40*	26.5 ± 19.4	26.3 ± 1.5	-	-	-	-	-	-	-	-
t-nonachlor	25	50*	34.3 ± 1.8	34.2 ± 8.1	-	-	-	-	25	25	16.8 ± 2	27.8 ± 3.5
γ-HCH	0.5	50	27.5 ± 2.7	40.7 ± 3.5	-	-	-	-	50	75	61.2 ± 2.9	65.4 ± 13.7
Dieldrin	6.25	50	22.4 ± 11.4	59.6 ± 1.9	-	-	-	-	-	-	-	-
BDE-47	0.25	20*	3.1 ± 0.5	17.9 ± 2.7	1.25	12.5	ND	55.3 ± 7.2	-	-	-	-
BDE-99	0.25	10	5.2 ± 1.9	36.3 ± 1.5	-	-	-	-	-	-	-	-
HBCD	0.25	15	35.8 ± 63.9**	58.1 ± 3.1	-	-	-	-	-	-	-	-

LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; RPC_{Max} : observed efficacy expressed as a percentage of the cell response exposed to 15 pM, 650 pM and 150 pM TCDD, respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D, corresponding to the MOEC; ND: Not Determined. * Corresponds to the highest tested concentration. ** IC_{50} estimated beyond tested concentrations. -: no response.

Table S3: Four parameters obtained in the dose-response curves (Graphpad PRISM) of the 16 active AhR antagonists in the rat DR-H4IIE cells.

Parameters	BDE-47	BDE-99	HBCD	PCB-28	PCB-53	PCB-101	PCB-118	PCB-138
Bottom (%)	0.2 ± 7.05	16.9 ± 12.4	0	20.3 ± 7.4	8.2 ± 5.5	47.2 ± 7.4	67.2 ± 1.4	43.1 ± 1.5
Top (%)	100.7 ± 2.6	93.1 ± 3.3	80.7 ± 1.4	104.2 ± 1.8	107.6 ± 1.2	99.5 ± 1.3	104.2 ± 1.8	92.9 ± 2.1
HillSlope	0.9 ± 0.1	0.9 ± 0.2	1.3 ± 0.5	1.1 ± 0.2	1.1 ± 0.1	1.5 ± 0.3	2.2 ± 0.5	2.4 ± 0.6
IC_{50} (μM)	3.1 ± 0.5	5.2 ± 1.9	35.8 ± 63.9	6.8 ± 1.7	7.3 ± 1.2	17.9 ± 3.8	0.3 ± 0.05	0.6 ± 0.07
Parameters	PCB-153	PCB-180	HCB	*α*-Chlordane	*o*-chlordane	*t*-nonachlor	*γ*-HCH	Dieldrine
Bottom (%)	0	5.1 ± 14.7	33.01 ± 13.4	0	0	25.8 ± 5.8	23.2 ± 4.6	45.6 ± 11.5
Top (%)	89.9 ± 1.1	102.9 ± 3.2	74.6 ± 2.6	89.1 ± 1.2	101.4 ± 4.8	89 ± 1.0	94.4 ± 1.6	96.7 ± 3.3
HillSlope	1.4 ± 0.2	0.9 ± 0.2	1.1 ± 0.5	2.4 ± 0.4	2.2 ± 1.5	4.98 ± 0.9	1.8 ± 0.3	0.8 ± 0.3
IC_{50} (μM)	18.5 ± 2.8	7.4 ± 3.3	17.9 ± 11.6	28.3 ± 3.3	26.5 ± 19.4	34.3 ± 1.8	27.5 ± 2.7	22.4 ± 11.4

Table 2. AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of the total POP mixture and Cl containing sub-mixtures (Cl, Cl+Br, Cl+PFAA) in DR-H4IIE, DR-T47-D and DR-Hep G2 cells (n = 3, 0.4% DMSO).

Mixtures	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)
POP	125	1000	374 ± 52	52.5 ± 2.1	500	1000	ND	80.1 ± 5.8	250	1000	ND	86.6 ± 2.2
Cl	250	1000	562 ± 54	53 ± 0.9	250	2000	ND	59 ± 1.6	-	-	-	-
Cl+Br	125	2000	468 ± 38	64.6 ± 1.7	500	1000	534 ± 253	76.1 ± 3.9	-	-	-	-
Cl+PFAA	75	2000	461 ± 78	41 ± 1.3	500	500	243 ± 104	77 ± 3.8	500	500	ND	77 ± 3.8

LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; RPC_{Max} : relative response at MOEC expressed in % of the response of TCDD EC_{50} 15 pM, 650 pM and 150 pM TCDD (respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D) corresponding to the MOEC; x bl: fold blood levels; ND: Not Determined. -: no response.

3.2. Aryl hydrocarbon receptor activities of the POP mixtures

AhR – mediated agonistic activities. Exposure to the total POP mixture or to the six sub-mixtures described in Table S1 did not induce any significant ($RPC_{Max} \geq 10\%$) AhR agonistic response in any of the cell lines (data not shown).

AhR – mediated antagonistic activities. The total POP mixture triggered an AhR antagonistic response in all cell lines (Table 2, Figure 1A). At a concentration in the culture medium corresponding to the blood level, the total POP mixture did not interfere with the response of the cells to EC_{50} TCDD. In contrast, significant and dose-dependent antagonistic responses were already observed at concentrations of 125, 250 and 500 fold blood levels, respectively, for DR-H4IIE, DR-T47-D and DR-Hep G2, although the concentrations of all 29 compounds were below their respective LOEC at these levels or even at 1000 fold blood levels (Table S1). In DR-H4IIE, the POP mixture displayed a significantly high AhR antagonistic efficacy of 52.5 ± 2.1% at 1000 fold blood levels and an IC_{50} = 374 ± 52 fold blood levels, while in both human cell lines, a significant response was observed, but did not reach below 80%, making the calculation of an IC_{50} not possible.

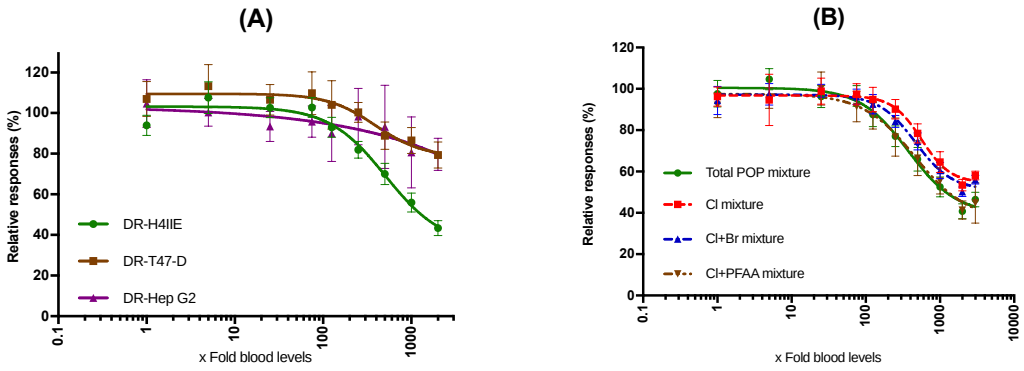


Figure 1. (A) Dose-response curves obtained from DR-H4IIE, DR-T47-D and DR-HepG2 cells co-exposed to 15 pM, 150 pM and 650 pM TCDD, respectively, and the total POP mixture. (B) Dose-response curves obtained from DR-H4IIE cells co-exposed to 15 pM TCDD, and the total POP mixture, or the Cl, Cl+Br and Cl+PFAA sub-mixtures (Mean ± SD, n = 3, 0.4% DMSO).

In parallel, six complementary sub-mixtures (PFAA, Br, Cl, Cl+Br, Cl+PFAA, Br+PFAA) were also tested to study the possible interactions between these groups of compounds. Antagonism was seen for all Cl containing mixtures (the total POP, Cl, Cl+Br and Cl+PFAA mixtures) in DR-H4IIE and DR-Hep G2, while only the total POP and Cl+PFAA mixtures showed responses in DR-T47-D (Table 2).

In DR-H4IIE cells, the three Cl containing sub-mixtures and the total POP mixture gave more or less similar responses with $IC_{50} \cong$ 400 to 500 fold blood levels and $RPC_{Max} \cong$ 50%. This indicates that the chlorinated compounds were responsible for the antagonism of all mixtures where they are present. Also, the antagonistic response curve of Cl+PFAA mixture overlapped that of the total POP mixture, which placed below those of the Cl and Cl+Br (Figure 1B). These observations suggest that the effect of the Cl mixture was somehow enhanced in the Cl+PFAA mixture, resulting in a dose-response curve overlapping that of the total POP mixture.

3.3. Predictions of the rat aryl hydrocarbon receptor antagonistic activities of the total POP mixture and Cl containing sub-mixtures

We evaluated the capacity of the three different mathematical models (concentration addition (CA), independent action (IA), and generalized concentration addition (GCA)) to predict the IC_{50} and dose-response curves of the total POP mixture in the most sensitive cell line, the DR-H4IIE.

Calculation of bottom and hillslope values. Because the 16 rat AhR antagonists contributed only 4.3% for the mass of the total POP mixture, along with considering all of the 29 POPs to calculating p_i, we also considered only the active compounds, subtracting the weight of the non-active compounds (sNA) as mentioned in section 2.2.3. WMBH method was unable to predict both bottom and hillslope values. AvBH and FoBH showed reasonable predicted values, especially AvBH and sNA FoBH for the total POP mixture and the three Cl containing sub-mixtures (Table S4).

Prediction of mixture effects of the three models. The results obtained after running the three models are shown in Figure 2. While the IA predicted a really strong response even at the lowest concentrations of the mixture, far out of the range of the measured curve, both the CA (CA-AvBH and CA-sNA FoBH) and GCA predictions resulted in calculated curves comparable to the measured curve. This refers that the 16 active compounds in the total POP mixture acted additively rather than independently.

The measured IC_{50} of the total POP mixture (374 ± 52 fold blood levels) was lower than the predicted value (784 fold blood levels for both the GCA and CA models), while the IA model predicted IC_{50} = 2153 fold blood levels. Thus, both CA and GCA models underestimated IC_{50} of the total POP mixture by about two folds, much less than one order of magnitude.

Concerning the calculated dose-response curves, the two additive models appeared to diverge: GCA produced a somewhat better prediction in the low concentration range, both CA

(CA-AvBH and CA-sNA FoBH) closely followed the experimental curve and only diverged at concentrations higher than around 1000 fold blood levels (Figure 2). Similar predictions were also shown for the three active Cl, Cl+Br, Cl+PFAA sub-mixtures (Table S4, Figure S2).

Table S4: Measured and predicted bottom, hillslope and IC_{50} of the CA models with several methods for the total POP mixture and Cl, Cl+Br, Cl+PFAA sub-mixtures.

Methods	Measured	WMBH	AvBH	FoBH	sNA WMBH	sNA FoBH
Total POP mixture						
Bottom (%)	52.5	5	21	0	100	0
Hillslope	1.3	0.5	1.7	34.2	-10.9	1.5
IC_{50} (x bl)	374	784	784	784	784	784
Cl sub-mixture						
Bottom (%)	53	83	25	0	100	0
Hillslope	2.1	6.9	1.8	2.1	-10.6	1.4
IC_{50} (x bl)	500	786	786	786	786	786
Cl+Br mixture						
Bottom (%)	64.6	83	21	0	100	0
Hillslope	2.1	7	1.7	2.1	-10.6	1.4
IC_{50} (x bl)	500	875	875	875	875	875
Cl+PFAA mixture						
Bottom (%)	41	5	25	0	100	0
Hillslope	1.4	0.4	1.8	38.6	-10.7	1.4
IC_{50} (x bl)	576	840	840	840	840	840

WMBH = weighted mean, AvBH = averaged, formulated hillslope and bottom (FoBH), sNA = substracted nonactive compounds. x bl: fold blood levels.

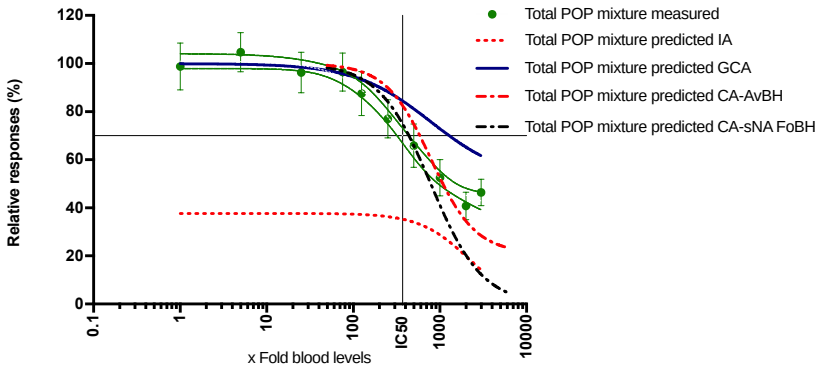
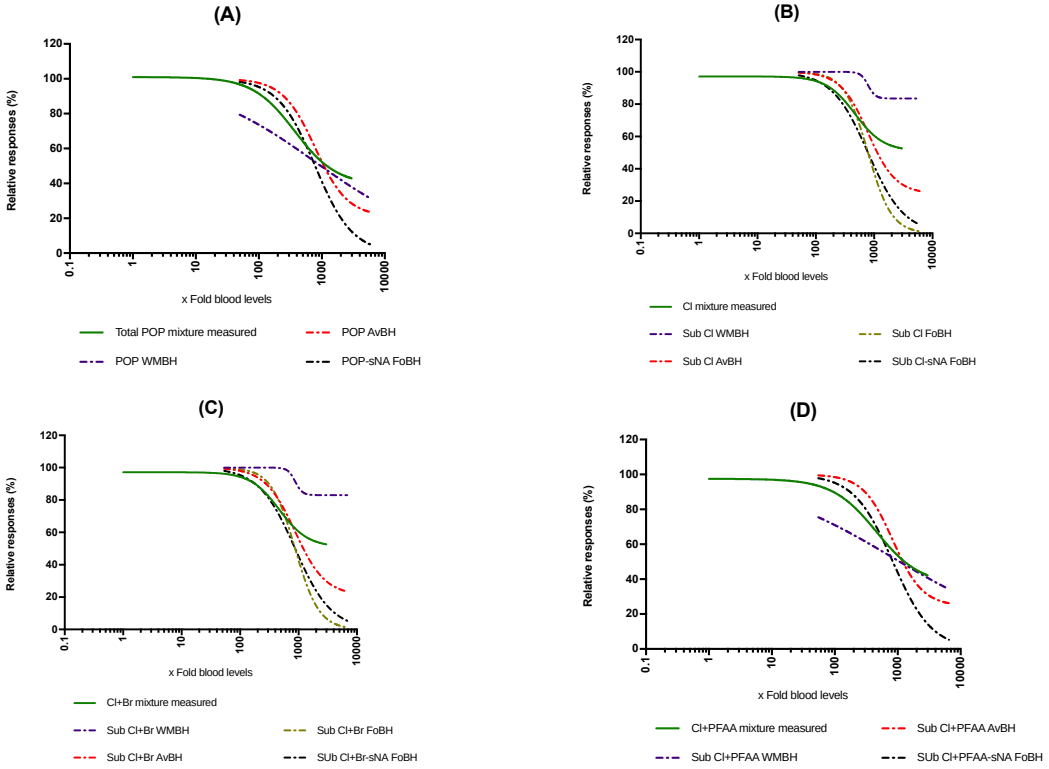


Figure 2. Measured and predicted dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture, and from three prediction models. CA = Concentration addition, IA = Independent action, GCA = Generalized concentration addition, AvBH = averaged hillslope and bottom, and sNA FoBH = subtracted nonactive compounds, formulated hillslope and bottom. Green dashed lines represent the 95% confidence interval of the measured response.



Figures S2: Predicted full dose-response curves of the concentration addition models with several methods to calculate the bottom and hill-slope for the total POP mixture (A) and Cl (B), Cl+Br (C), Cl+PFAA (D) sub-mixtures. WMBH = weighted mean, AvBH = averaged, formulated hillslope and bottom, sNA = subtracted nonactive compounds.

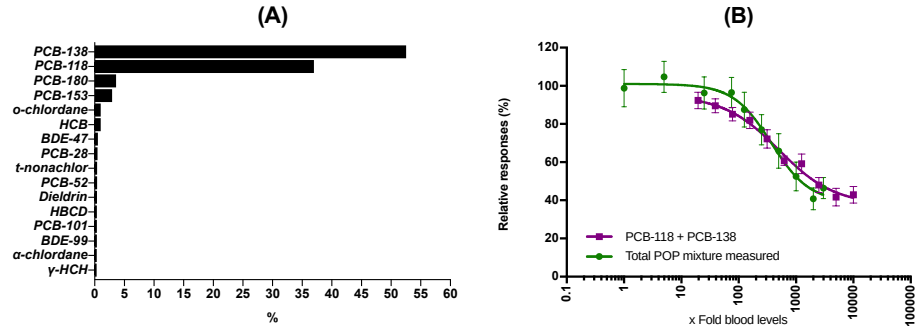


Figure 3. (A) Distribution of toxic units of the 16 active AhR antagonists. (B) Dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture or a binary mixture consisting of PCB-138 and PCB-118 (Mean ± SD, n = 3, 0.4% DMSO).

Toxic units. Deriving from CA model, toxic units (*i.e*. the ratio of the concentration partition of a compound to its IC_{50} ($p_i/IC_{50,i}$)) scales the concentrations of the mixture components to its toxicity, represented by the transcriptional activity of the Cyp1a1 promotor. Thus, it has been applied to identify the main driver(s) for mixture effects in CA model[24,28,41]. Figure 3A clearly shows that PCB-138 and PCB-118 were the two main contributors for the AhR antagonism of

the total POP mixture, constituting to 90% of the total combined activity. Since they were also partial agonists, it is likely that, in the presence of TCDD, they behaved mainly as antagonists especially at low concentrations. Following this prediction, a binary mixture of PCB-138 and PCB-118 was generated according to their concentration in the total POP mixture. The dose-response curve of this mixture followed that of the total POP mixture very closely, with an IC_{50} of 505 ± 67 fold blood levels (while IC_{50} = 374 ± 52 fold blood levels for the total POP mixture) (Figure 3B).

4. Discussion

AhR transactivation activities of the 29 POPs and the mixtures. This study shows that a majority of the chemicals composing the realistic total POP mixture are actually AhR antagonists (16 in DR-H4IIE, five in DR-Hep G2 and seven in DR-T47-D cells). As expected, the total POP mixture and the Cl containing mixtures were also shown to be antagonistic. These activities were AhR-dependent, and seemed to act through competition for the TCDD binding site, except for *t*-nonachlor and α-chlordane.

In our study, we tested the AhR transcriptional activity of POPs and POP mixtures using a transcriptional reporter assay, which basically reports the canonical AhR-driven pathways via AhR-ARNT-DRE interactions. However, we observed that two of the compounds (*α*-chlordane and *t*-nonachlor) do not seem to exert their antagonistic effect through competitive binding to AhR. Several possible non-canonical AhR-driven pathways could contribute to the observed results, such as crosstalk with other nuclear receptors, regulation of cell cycle and MAP kinase cascades, or novel AhR DNA-binding partners[42,43]. Further studies of these mechanisms are required, but were outside of the scope of this paper.

Our results concerning single compound testing are in general consistent with previously published studies, where available. For agonistic activity, PCB-118 displayed highest RPC_{Max} = 61.3% at 50 µM, with EC_{50} = 25 ± 13 µM similar to previously finding (9.3 ± 2.5 µM)[44]. EC_{50} = 4 ± 0.8 µM of BDE-99 was lower than previous report (EC_{50} > 15 µM)[45]. For PCB-138, we observed an agonistic effect with a high EC_{50} = 28 ± 6.4 µM, which was not reported before[44] (Table S2).

For antagonistic activities, in this study, IC_{50} of PCB-28 and PCB-138 were 6.8 ± 1.7 and 0.6 ± 0.07 µM (Table 1), close to previous estimates of 9.0 ± 2.9 µM and 1.4 ± 0.1 µM, respectively[44]. BDE-47 activity (IC_{50} = 3.1 ± 0.5 µM) was similar to those previously reported (2.7 ± 0.7 µM)[45] and (3.7 ± 0.8 µM)[44]. However, the IC_{50} = 5.2 ± 1.9 µM for BDE-99 found in this study was lower than that previously reported (13 ± 0 µM)[45]. This study reports for the

first-time data for the AhR transcriptional activity of the 29 POPs in both human mammary gland carcinoma DR-T47-D and hepatoma DR-Hep G2 cells.

Differences in our IC_{50} or EC_{50} values and in those in the previous findings[44,45] may result from differences in the experimental design and the regression methods (*i.e.* the number of concentration points, availability of a maximum effect if a full curve is generated, extrapolation if the maximum effect is not reached, and the regression function used with either four or three parameters). Our study not only confirms and consolidates previous findings[44,45], but it also contributes new data including full dose-response curves with four parameter fit (see Table S3) which can be used for further data treatment or calculation of the joint effect of any mixture made from these 29 POPs for the rat DR-H4IIE cells.

We observed species and tissue differences in the AhR transcriptional activities of the individual POPs and of the mixtures. In general, the rat DR-H4IIE cells were more sensitive than the two human cells towards the effects on AhR transactivation when exposed to individual POPs or POP mixtures. Several considerations may explain this result. Interspecies differences in AhR structure will obviously shape the sensitivity. Rats are 1000 folds more sensitive to TCDD than guinea pigs[46]. Mouse AhR has a higher affinity than human AhR due to the different position of the important amino acid Valine (V381 in humans corresponding to V375 in mice)[47]. Human AhR has shown a higher relative affinity for certain structurally compounds *i.e.* endogenous ligands or polyphenols[48]. Moreover, the difference in genetic modification (origins of the integrated promotor and DREs) of the rat DR-H4IIE compared to the two human cell lines could also play a role for the specific responses. Differences in regulatory processes downstream of AhR binding may be responsible, such as differential binding to transcriptional coactivators[49]. Finally, in the antagonistic tests, the POPs have to compete with increasing TCDD concentration (15 pM, 150 pM and 650 pM, respectively for DR-H4IIE, DR-T47-D and DR-Hep G2), which could lead to the lower sensitivity to detect an antagonistic activity of the POPs in the two human cells compared to the rat cells.

Mixtures relevant for human exposure antagonize AhR activation. The most striking result of this study is that the total mixture of 29 POPs, derived from concentrations found in the blood of a Scandinavian population, and sub-mixtures thereof were found to exert only antagonistic effects on AhR. This observation is consistent with our results obtained from testing each compound alone, revealing a majority of antagonistic compounds. AhR antagonism of POPs has been observed in several screening studies[44,45] and mixture studies[50,51]. However, while the AhR agonistic activity of POPs has been studied for decades,

the antagonistic counterpart has not yet received much attention, especially regarding its physiological consequences on an organism's health.

This finding challenges the method of using toxic equivalency factors (TEFs) and toxic equivalent quantities (TEQ) for risk assessments of mixtures of AhR ligands. The World Health Organization assigned TEFs for PCDDs/PCDFs/dl-PCBs, expressed as relative effect potencies compared to the most toxic form TCDD. Regarding their additive mechanism, TEFs are also used to estimate TEQ for a mixture of compounds by adding up the TEF fraction and the concentration of each compound within the mixture[52]. However, PCB mixtures alone or in combination with PCDDs/PCDFs (usually TCDD) have shown additive but also non-additive responses[53,54]. Also, several environmentally abundant biphenyls antagonize the *cyp1a1* induction by TCDD[12], while some dl-mono-ortho-substituted PCBs revealed both agonistic and antagonistic properties[55,56]. In many environmental samples, the ratio between PCBs and AhR agonists is above 1000, indicating that antagonisms, resulting from interactions between AhR agonists and PCBs[57], are not irrelevant. Therefore, the antagonistic effect of these compounds should be also considered to calculate the effect of mixtures. Since they are more abundant in real-life mixtures, their antagonisms may undermine or even abolish the overall dioxin potency of the environmental mixtures[12].

Our finding also raises the issue of the biological significance of a predominantly AhR antagonistic mixture in the blood of a human population. We found antagonistic activities at levels of 125 fold blood levels in rat liver cells or 250 or 500 folds in human mammary gland and liver cells, levels that may realistically be reached after an accident or in exposed populations.

It is important to note that in the total POP mixture used here[36], no dioxins and dl-compounds were included. That allows us to study the antagonism of the human-exposure relevant POP mixtures by isolating them from the dioxin and dl-compounds. But the roles of the dioxin and dl-compounds in human exposure should have also been considered. Therefore, we attempted to estimate the effect of the total POP mixture in a real-life situation. According to Kvalem *et al*.[58] , the median of dl-compounds in Norwegian human blood was 33.1 pg TEQ/g lipid, which is equal to 0.6 pM in blood assuming that blood contains 0.6% fat and 1 ml blood = 1 g. Thus, the respective LOECs (for the POP mixture AhR antagonistic activity) in DR-H4IIE, DR-T47-D and DR-Hep G2 of 125, 250, and 500 fold human blood level correspond to 75 pM, 150 pM and 300 pM of dl-compounds, respectively. This concentration is close to the TCDD EC_{50} (15 pM TCDD in DR-H4IIE, 150 pM in DR-T47-D and 650 pM in DR-Hep G2 cells)

used in our antagonistic assays. Therefore, it is likely that the total POP mixture would antagonize the activity of these dl-compounds in the Scandinavian population.

Furthermore, the question arises whether an overall AhR antagonistic exposure would actually cause health problems by interfering with the normal AhR function. Increasing evidence suggests that endogenous AhR ligands exist[59,60], complemented by dietary phytochemical-derived AhR agonists/antagonists[61,62]. AhR induced functions are essential for a variety of normal physiological functions. In mammary tissue, AhR likely plays a physiological role in coordinating development, differentiation, cell growth, and signaling of hormones[63–67]. Knock out mice or mice with low affinity AhR variants display impaired survival, growth, fertility, liver function and innate and adaptive immunity[68]. It is thus conceivable that the presence of highly stable POPs may interfere with the essential function of AhR controlled by mostly short-lived endogenous and dietary ligands, and thus impair cellular AhR mediated processes. The risk caused by AhR antagonism as the major effect of POPs could well exceed that due to their agonistic effect, however the health effect associated to AhR antagonism is unclear and deserves further investigation.

Predictions of mixture effects. Risk assessment for mixture exposure is crucial to protect the health of both humans and wildlife. The individual chemical approach underestimates the mixture exposure and decreases the accuracy of risk assessment[22,23,69]. In addition, the risk of exposure to multiple chemicals at doses below their threshold, which is the most common case in real-life, should not be underestimated or assumed as no-effect.

In this study, the best prediction results were obtained using the CA (concentration addition) and GCA (generalized concentration addition) models. They performed well in predicting $IC_{mix,50}$ = 784 fold blood levels within two folds from the measured value (374 fold blood levels). This is considered as well accepted in predicting the combined effect of complex mixtures *i.e.* the total POP mixture with its components present at low concentration (lower than their LOECs at 1000 fold blood levels) and belonging to different compound groups.

CA is often chosen as the default model[70] for predicting mixture activities, first for mixtures with similar compounds[24] then expended to dissimilar compounds[23,25,26]. Previous studies have shown the capability of the CA model to predict the mixture effect using the information of individual chemicals obtained *in vitro*[25,41,71,72], *ex vivo*[73] or *in vivo*[23,25]. *In vitro* research has mainly focused on an equimolar mixture with less than ten components and at high exposure concentrations. Birkhøj *et al*.[25] successfully applied the CA model to predict the antiandrogenic effect of a mixture of five commonly used pesticides at 10 μM each. In contrast, the CA model

was unable to predict the effect on thyroid hormone function and AhR transactivation of another mixture of five different pesticides at the maximum concentration of 50 μM each, due to the presence of an inhibitory compound[71]. Other studies focused on more complex mixtures with multiple components at lower doses, typically below their threshold doses, or on human or environmentally relevant exposure scenarios. Two complex mixtures of 17 estrogenic chemicals were screened for estrogenic activities, reporter-gene (ERLUX) and cell proliferation (ESCREEN) endpoints[41]. This represents one of the most comprehensive studies on the effects of mixtures where they were able to predict the effects of the two mixtures.

GCA, on the other hand, has been recently developed and proven useful specifically for calculating mixtures containing partial agonists[30–32], but has not been applied before to calculate the activity of AhR antagonists. It allows to consider theoretically the fact that some agonists never reach the full activity of TCDD, or that some antagonists present partial agonistic activities.

The difference between CA and GCA models resides in the predicted dose-response curve of the mixture and in the maximum predicted activity of the total POP mixture (Figure 2). GCA predicting the mixture response, allows to generate the full dose-response curve of the mixture using only the data from testing the individual compounds (*i.e.* RPC_{Max}, concentration and IC_{50}). The reason why this predicted curve diverged from the experimental curve at higher concentrations could result from the assumption that the hillslopes of all components, and so of the POP mixture, are equal to 1, which is clearly not the case (Table S3). However, GCA predicted the bottom (RPC_{Max} = 52%) very close to the observed value for the total POP mixture (52.5%) thanks to its consideration of the RPC_{Max}.

On the other hand, CA provides a prediction of $IC_{mix,j}$ without the full dose-response curve. Therefore, we calculated the hillslope and the bottom values for the mixture response based on its components by formulating several possibilities. The dose-response curve generated by CA with averaged bottom hillslope (CA-AvBH) resulted in the best fit with reasonable hillslope and bottom values (1.7 and 21%) compared to 1.3 and 52.5%, respectively of the measured POP mixture curve. Subtracted non-activated compounds and formulated bottom hillslope (sNA FoBH) predicted an overlapped curve with the observed up to 1000 fold blood levels because of its closer H_{mix} = 1.5, but overestimated the extension of antagonism at higher concentration, leading to the prediction of B_{mix} = 0% for the total POP mixture. B_{mix} is important when predicting the activity of a mixture for risk assessment. Therefore, CA-AvBH rather than CA-sNA FoBH was chosen as a more suitable prediction in our case. The CA model also provides a good prediction of $IC_{mix,50}$ for the response of the DR-H4IIE cells to the active sub-

mixtures as to the total POP mixture, and reasonable predicted dose-response curves (Table S4, Figure S2).

Finally, IA (independent action) was designed specifically for mixtures of compounds with clearly different MOAs to combine probabilities of action of individual compounds. Previous studies showed that IA outperformed CA[23] or was comparable to CA with equal[29] or not more than five-fold differences[28,74] in predicting the combined effects for chemicals having different MOAs. The bad performance of IA to predict either IC_{mix} or the dose-response curve clearly results from the mixture studied here, where we showed that most components act through the same MOAs. At low doses, accumulation of the individual, low probabilities derived for a high number of individual compounds presumably resulted in the dramatic overestimation of the antagonistic effect.

Conclusions

We tested the AhR agonistic and antagonistic activities of 29 POPs shown to contaminate human blood, both in individual and mixture forms. AhR transactivation activities in three reporter cell lines exposed to the 29 POPs and the mixtures were different due to the species and tissue-specific responses. The predominant individual activities of the POPs were AhR antagonism, as shown for 16 compounds out of 29 in rat DR-H4IIE cells, and for seven and five compounds in human DR-T47-D and DR-Hep G2, respectively. The total POP mixture already induced a significant AhR antagonistic activity at concentrations of 125, 250, and 500 fold human blood levels, respectively in DR-H4IIE, DR-T47-D and DR-Hep G2, although each individual compound was present at concentrations lower than its LOEC at these levels. Such blood levels of POPs could realistically occur in food or environmental contamination incidents or in highly exposed sub-populations. Chlorinated compounds, among which PCB-118 and PCB-138 contributed 90% to the activity of the total POP mixture, were the drivers for AhR antagonism in DR-H4IIE cells. Finally, CA and GCA proved to be good tools to predict the mixed effect of the total POP mixture with only two-fold underestimated IC_{50} and acceptable does response curves. Hence, the compounds acted additively in the mixtures. Although limitations remain to fully describe the effects of realistic mixtures due to biological complexity, the predictions obtained using CA and GCA seem suitable for establishing general regulatory guidelines for mixture toxicity assessments. In addition, the data generated in this study for individual compounds will be useful to predict the effect of other complex mixtures constituted by these compounds.

Conflict of interest: The authors declare that there is no conflict of interest.

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Chapter 3

As mentioned before, the Aryl hydrocarbon receptor (AhR) is involved in xenobiotic metabolism and controls many biological pathways. AhR functions can be dysregulated by inappropriate ligand activation or inhibition, leading to subsequent health concerns. Recently, AhR physiological roles have gained more attention since AhR is activated by a wide range of structurally diverse endogenous and exogenous compounds. Among them, 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan derivative is considered the highest affinity AhR ligand to date which is produced endogenously by the host or by the symbiotic microbiota, while polyphenols represent the largest source of AhR natural modulators in the human diet.

These new findings prompted us to compare the responses of human and rat AhR towards endogenously produced (FICZ) versus exogenous (TCDD) AhR ligands. Furthermore, we wanted to investigate different subclasses of natural AhR modulators, the polyphenols, that a human population could be in contact with.

Thus, in chapter 3, we studied the AhR transactivation by FICZ/TCDD *in vitro* in a time-dependent and species-specific manner using CALUX assays based on two cancer cell lines deriving from rat (DR-H4IIE) and human (DR-HepG2) hepatoma. The aims were to: (a) study species-specific (rat versus human) responses of AhR to FICZ or TCDD in the presence or absence of endogenous ligands (by comparing medium with Trp versus without Trp, Trp being the major source of AhR endogenous ligands), (b) evaluate the effect of polyphenols, and a mixture thereof, either alone or co-exposed with FICZ or TCDD to mimic human realistic exposure to mixtures of AhR modulators, and (c) reveal the most active polyphenol(s) and possible interactions among these compounds in the mixture.

The results obtained have been submitted to Environmental Pollution.

In vitro differential responses of rat and human aryl hydrocarbon receptor to two distinct ligands and to different polyphenols

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Abstract

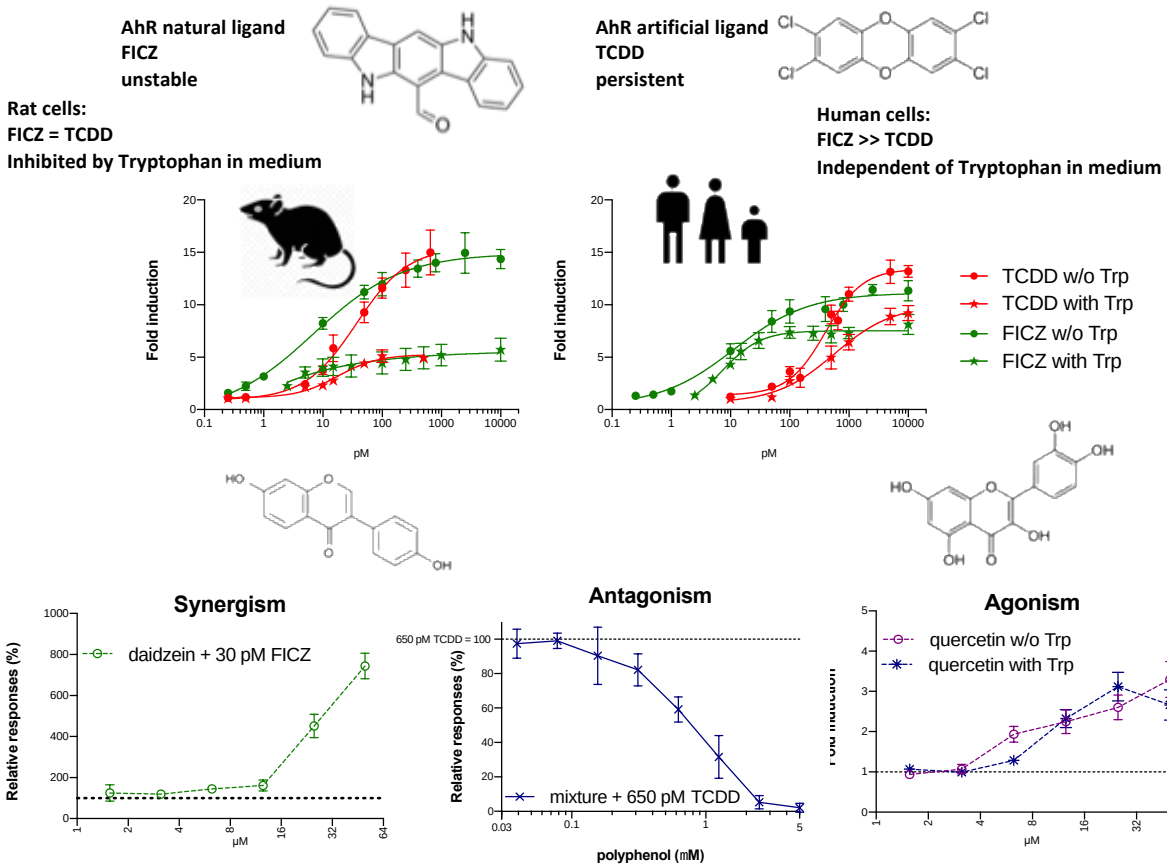
TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and several other environment/food-borne toxic compounds induce toxicity via aryl hydrocarbon receptor (AhR). AhR is also modulated by various endogenous ligands *e.g.* highly potent tryptophan (Trp)-derivative FICZ (6-formylindolo[3,2-b]carbazole) and natural ligands abundant in the human diet *e.g.* polyphenols. Therefore, evaluating AhR responses and its activity in different species, is crucial for understanding the physiological functions of AhR, establishing a risk assessment, and exploring further applicability of AhR mediators in drug and food industry towards human-based usages. We studied the AhR transactivation of FICZ/TCDD *in vitro* in a time-dependent and species-specific manner using two DR-CALUX assays derived from the rat (DR-H4IIE) and human (DR-HepG2) hepatoma cancer cell lines. We observed for the first time that FICZ potency was similar in both cell lines, but FICZ was 40 times more potent than TCDD in human cells. Depleting Trp-derivative endogenously produced ligands by using culture medium without Trp, resulted in 3-fold higher AhR activation upon adding FICZ in rat cells, in contrast to human cells which revealed a fast degradation of FICZ from 10h induction to complete disappear after 24h. Seven polyphenols and a mixture thereof, chosen based on commercially recommended doses and adjusted to human realistic exposure, caused rat and human species-specific AhR responses. Two isoflavones (daidzein and genistein) induced a rat AhR synergistic effect with FICZ and/or TCDD EC_{50}, while quercetin, the flavones (baicalin and chrysin), curcumin, and the mixture exerted a strong human AhR inhibitory effect. The non-flavonoid resveratrol acted as an agonist on both cell lines. Strikingly, resveratrol and

quercetin at their realistic nanomolar concentrations acted additively in the mixture to abolish human AhR activation in presence of various TCDD concentrations. Taken together, these results illustrate the species-specific complexity of AhR transactivation by various ligands and highlight the need for studies of human-based approaches.

Keywords: Aryl hydrocarbon Receptor; rat and human; polyphenols; quercetin; antagonisms

Capsule: Diet-borne endogenous and exogenous AhR modulators act differentially in human and rat hepatoma with quercetin and resveratrol being the strong human AhR antagonists.

Graphic abstract:



Highlights:

- Tryptophan-derivative FICZ is 40-fold more potent than dioxin to induce human AhR.
- FICZ-induced human AhR activation is completely vanished at 24h post-exposure.
- Rat AhR responds strongly to endogenously produced ligands derived from tryptophan.
- Daidzein and genistein synergize with FICZ and/or dioxin in activating rat AhR.
- Quercetin and resveratrol inhibit dioxin-activated human AhR at realistic doses.

1. Introduction

Aryl hydrocarbon receptor (AhR) is known for its role as a xenobiotic mediator[1]. Environment/food-borne contaminants like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce their toxicity via the AhR signaling[2,3]. Ligand (TCDD)-activated AhR undergoes a translocation from cytosol to nucleus and binds to its heterodimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT). This AhR/ARNT heterodimer binds to DNA sequences called dioxin responsive elements (DREs) to activate the expression of AhR inducible genes, including the phase I drug-metabolizing enzymes, the cytochrome p450 (CYP) family (*e.g*. *CYP1A1*). Therefore, the methods measuring DRE–regulated gene expression are widely accepted for determining AhR activation[4], among which cell-based screening methods such as Chemically Activated LUciferase gene eXpression (CALUX) are the most common[5].

The Ah receptor is also activated by a wide range of diet-borne endogenous and natural ligands to regulate several essential physiological functions in development and homeostasis, including immune responses, cell differentiation and proliferation, reproduction, and tumor suppression[6]. Indeed, AhR is considered as a sensor, connecting external environmental signals to cellular processes[6,7]. Catabolism of the essential amino acid tryptophan (Trp) either by the host or by the symbiotic microbiota yields numerous physiologically relevant AhR ligands[8,9]. Among them, 6-formylindolo[3,2-b]carbazole (FICZ) is considered the highest affinity AhR ligand to date with an estimated dissociation constant K_d of 0.07 nM, which is significantly lower than the TCDD K_d of 0.48 nM, measured in a competitive binding assay performed in rat hepatic cytosol[10]. It is produced by photochemical reactions under ultraviolet and visible light[11,12] or by enzymatic and oxidative pathways[11,13], thus ubiquitously distributed in most cell types[13] to perform several essential functions especially in immunity[14]. FICZ is able to activate AhR in various species such as fish[15], frog[16], and birds[17,18]. However, little is known about its potency and mode of action compared to the widely studied AhR ligand TCDD.

On the other hand, polyphenols represent the largest source of natural AhR modulators in the human diet[19]. They are secondary metabolites of plants with a common polyphenol structure and diverse chemical entities[20]. They are popular in research and food manufacture due to their significant antioxidant ability which could be useful to prevent various diseases associated with oxidative stress *e.g.* cancer, cardiovascular, and neurodegenerative diseases[21,22]. Besides, they are also able to modulate the activity of several enzymes and receptors[23]. For example, polyphenols have been shown to affect AhR activity in several ways including directly as AhR functional agonists or antagonists, indirectly by interfering with AhR

translocation, AhR-ARNT heterodimerization, AhR-DNA binding, or altering co-activators and co-repressors[19].

AhR normal functions can be dysregulated by inappropriate ligand activation or inhibition[7], leading to subsequent health concerns[19]. Thus, AhR signaling needs to be tightly controlled by several mechanisms. For example, different susceptibility to metabolic feedback likely determines the mode of action of TCDD and FICZ *in vivo*[24]. AhR ligands, except metabolically stable ligands such as TCDD, activate AhR to induce their own oxidative metabolism via induction of AhR downstream genes *e.g.* CYP1 isoenzymes, and subsequently, their clearance from the body[25]. TCDD induces persistent AhR responses, while FICZ, which is rapidly metabolized by CYP1 isoenzymes[11,26], causes only transient AhR signaling. Moreover, while most of the risk assessment data for humans are based on rodent models, there are large interspecies differences in susceptibility to toxic effects induced by dioxins[27], and potentially by these endogenous and natural AhR ligands. Therefore, evaluating AhR responses depending on exposing species is crucial to understanding the physiological functions of this receptor as well as to establish the risk assessment and further applicability of AhR endogenous and natural mediators in drug and food industry towards human-based usages.

In this study, reporter gene assays involving two cancer cell lines derived from, respectively rat and human hepatoma were used to study the effect of FICZ versus TCDD and seven commonly commercialized polyphenols on the transcriptional activity of AhR. The aims were to: (a) examine species-specific (rat versus human) responses of AhR to FICZ or TCDD in the presence or absence of endogenous ligands (by comparing medium with Trp versus without Trp, Trp being the major source of AhR endogenous ligands), (b) evaluate the effect of polyphenols, and a mixture thereof, either alone or co-exposed with FICZ or TCDD to mimic human realistic exposure to mixtures of AhR modulators, and (c) reveal the most active polyphenol(s) and possible interactions among these compounds in the mixture.

2. Materials and methods

2.1. Chemicals and suppliers

FICZ was bought from Sigma Aldrich (Missouri, USA), while TCDD in DMSO was obtained from Wellington Laboratories (Ontario, Canada). Seven polyphenols: daidzein, quercetin, resveratrol, genistein, baicalin, curcumin, and chrysin were selected based on their common commercial availability as food supplements. They were purchased from Sigma Aldrich and dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Molinons, France). Their detailed description is given in Table S1. A mixture of these seven polyphenols was designed to reflect

Table S1. Seven selected polyphenols.

Polyphenols	Purchase reference (Sigma)	Classification	Commercial sellers (food supplements)	Recommended uptake dose (food supplement) (mg/day)	Theoretical blood level (μM)
Quercetin	Q4951	Flavonols	Nature's Best, UK	500	0.33
Resveratrol	R5010	Stilbenes	Pureclinica, UK	500	0.44
Curcumin	78246	Hydroxycinnamic acid	Nature's Best, UK	1000	0.54
Chrysin	95082	Flavones	Swanson, UK	500	0.39
Baicalin	572667	Flavones	Supersmart, Luxembourg	1000	0.45
Genistein	G6649	Isoflavones	Vital Nutrients, USA	500	0.37
Daidzein	D7802	Isoflavones	Ultimate Nutrition, USA	100	0.079

the realistic mixture exposure scenario of humans based on food supplement consumption or via their diet. The mixture contained each polyphenol at a concentration corresponding to a theoretical blood level arbitrarily calculated considering that the recommended ingested dose is diluted in five liters of blood, with an additional 1000-fold dilution factor to consider the intestinal absorption and the high metabolic rate of polyphenols. The polyphenols had nanomolar as their concentrations in the mixture at blood level (Table S1), relevant to human exposure[28]. To make up the mixture, after dissolving each compound individually in ethanol (Merck, Massachusetts, USA), the ethanolic solutions were pooled together to obtain the desired concentrations of the polyphenols in the mixture. The ethanol was removed under nitrogen flow to near dryness. Then the mixture was left overnight at ambient temperature and protected from light to complete dryness. DMSO was added to the dried residue to obtain the final solution. The stock solutions and working solutions of the polyphenols and the mixture were stored at -20$_{o}$C and protected from light. Six successive two-fold dilutions were prepared from the stock solution right before use to obtain the tested concentrations which did not exceed 50 μM for the polyphenols and 65 μM for the mixture.

2.2. Determination of aryl hydrocarbon receptor transcriptional activities

2.2.1. Cell lines and DR-CALUX assays

Two dioxin responsive (DR) hepatoma cell lines were used, rat DR-H4IIE from BioDetection System (Amsterdam, The Netherlands), and human DR-HepG2 previously produced within in the laboratory of Prof. Scippo (Liege, Belgium)[29]. These cells were stably transformed with a vector containing four DREs, either from the mouse *Cyp1a1* promotor for the rat cells or synthetic for the human cells, upstream of, respectively the mouse mammary tumor virus promoter[30] or the thymidine kinase promoter[29] which control the transcription of a luciferase reporter gene. The cells were cultivated in MEMα supplied by Thermo Fisher Scientific (Massachusetts, USA) with 10% v/v fetal bovine serum (Greiner, Kremsmünster, Austria) and 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma Aldrich). The cells were maintained in a water saturated atmosphere containing 5% CO_2 at 37°C.

We used the DR-CALUX assays to study the AhR transcriptional activity of FICZ compared to TCDD and of the polyphenols and the mixture alone or co-exposing with TCDD/FICZ EC_{50} (more details about each experiment see section 3). The assays were carried out by following the method described elsewhere[5,29]. Briefly, after 24h of seeding in white clear-bottomed 96 well microplates (Greiner) and reaching 90% confluence, the cells were exposed to the test compounds/mixture for various times in medium with Trp (MEMα, 10mg/l L-Tryptophan) and without Trp (DMEM w/o Trp, Biomol, Hamburg, Germany). For exposure, the culture medium

was replaced by freshly prepared medium with test compounds and exposure was carried out with protection against direct light. After exposure, the cells were washed with phosphate buffered saline (Sigma Aldrich) and lysed by a solution containing Triton X100 (Sigma Aldrich). Light production was triggered by adding luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) to the cell lysate and measured by a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). Each experiment was repeated at least three times independently in triplicate wells, together with a reference curve of TCDD/FICZ for quality control. The final DMSO concentrations were 0.1% and 0.2%, respectively for the activation and the co-exposure experiments. Cytotoxicity was assessed by visual inspection of cell morphology and attachment under microscope.

2.2.2. Calculations of fold induction (FI), relative response (RR), EC_{50}, and IC_{50}

Luciferase activities in the reporter gene assays were recorded as relative light units (RLU). The final results in the activation tests were presented as fold induction (FI). In the co-exposure tests which mainly focused on the inhibition potency of the compounds/mixture to the AhR activities of the spike-in FICZ/TCDD EC_{50}, the results were presented as relative response (RR):

$$(1)\ FI_{i(activation)} = \frac{RLU_i}{RLU_{DMSO}}$$

$$(2)\ RR_{i(co-exposure)} = \frac{RLU_i - RLU_{DMSO}}{RLU_{TCDD/FICZ} - RLU_{DMSO}}$$

The fold induction (FI_i) in an activation test was the ratio (fold) between the response of the tested compound/mixture (i) (RLU_i) and the response of DMSO in the same plate (RLU_{DMSO}) (Eq. (1)). The relative response (RR_i) in a co-exposure test was the percentage (%) of the response of the cells to the tested compound/mixture (i) compared to the spike-in TCDD/FICZ EC_{50}, subtracting from the baseline DMSO response (Eq. (2)). Non-linear regressions of the four-parameter equations for agonistic activation (Eq. (3)) or antagonistic inhibition (Eq. (4)) effects (FI or RR, respectively) were used to fit the dose-response curves (Graphpad PRISM software, version 7, San Diego, California, USA):

$$(3)\ FI_{i(activation)} = bottom + \frac{x^{hillslope}(top - bottom)}{x^{hillslope} + {EC_{50}}^{hillslope}}$$

$$(4)\ RR_{i(co-exposure)} = bottom + \frac{top - bottom}{1 + \frac{x^{hillslope}}{{IC_{50}}^{hillslope}}}$$

where *x* was the concentration of the tested compound/mixture. The corresponding EC_{50} and IC_{50} were the concentrations of the tested compound/mixture exerting half the maximal effect[31]. Data from tests revealing cytotoxicity were excluded from the analyses.

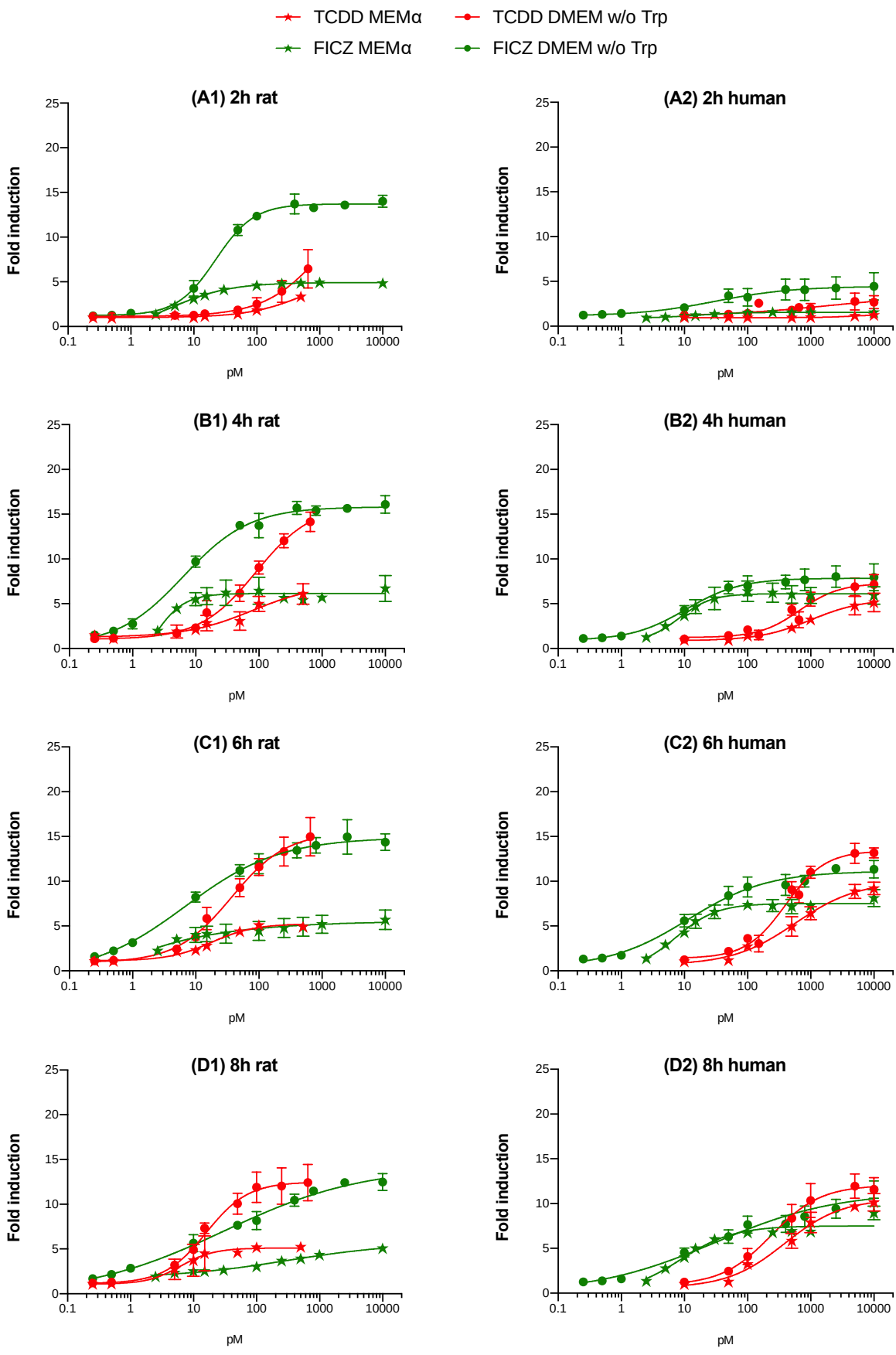
3. Results

3.1. Rat and human aryl hydrocarbon receptor transcriptional activation of FICZ/TCDD

In a first experiment, the dose-dependent AhR transcription activated by FICZ and TCDD was determined in a time series (2h – 24h) in rat DR-H4IIE and human DR-HepG2. To investigate the role of endogenously produced AhR ligands, we compared the cells' response in the standard medium containing Trp (MEMα) and in the medium without Trp (DMEM w/o Trp), with Trp being the main precursor of these compounds. FICZ strongly induced the AhR transcriptional activity, to a similar extent (FI_{max} = 10- to 15-fold at 250 pM FICZ) compared to TCDD in both cell lines, however with different response patterns (Figures 1 and 2).

The response of the rat DR-H4IIE to FICZ and TCDD was influenced by the presence of Trp in the culture medium. In MEMα, rat AhR activation was low (the maximum fold induction FI_{max} ≅ 5-fold induction until 10h exposure) with a gradual increase for both TCDD and FICZ exposure, reaching a maximum of 12 ± 0.2 and 9 ± 0.2 fold, respectively, after 24h exposure (Figure 2A1). In DMEM w/o Trp, although the cells responded faster and more strongly to FICZ than to TCDD, reaching fold induction as high as 14 ± 0.4 already after 2h exposure (Figure 1A1), their response to both FICZ and TCDD after 4h exposure was comparable (Figure 1B1) and remained similar until 24h exposure (Figure 1G1), although the dose-response curves of rat AhR response to FICZ started to degrade after 8h induction with much steeper curves. Both FI_{max} and EC_{50} remained relatively similar for FICZ and TCDD (Figure 2-1).

In contrast, the AhR transcriptional activation of the human DR-HepG2 mainly depended on the added agonist (FICZ or TCDD), largely irrespective of the presence or absence of Trp in the culture medium. Human AhR activation induced by TCDD in both culture media increased over time and reached a maximum of 21-fold induction after 24h exposure (Figures 1G2 and 2A2). In contrast, the response activated by FICZ increased during the first 4h exposure, remained stable between 4h and 8h exposure, then decreased and completely vanished after 24h exposure (Figure 1-2). The rapid decrease of FICZ-induced AhR activation (resulting in steeper curves and low FI_{max}) after 10h exposure indicated a fast metabolism of FICZ in the human cells. The human cells were also dramatically more sensitive to FICZ than to TCDD. For example, in DMEM w/o Trp, FICZ elicited the maximal 11-fold induction after 6h exposure to 250 pM FICZ, while the cells at this time required 10,000 pM of TCDD (Figure 1C2) to have the same effect, indicating a 40-fold higher potency of FICZ compared to TCDD. The FICZ EC_{50} in human cells (exposed in DMEM w/o Trp) were 11 ± 2.4 pM, ~40-fold lower than TCDD EC_{50} = 403 ± 29 pM after 6h exposure (Figure 2B2).


TCDD MEMα
TCDD DMEM w/o Trp
FICZ MEMα
FICZ DMEM w/o Trp
(A1) 2h rat
(A2) 2h human
(B1) 4h rat
(B2) 4h human
(C1) 6h rat
(C2) 6h human
(D1) 8h rat
(D2) 8h human
Fold induction
pM
0.1
1
10
100
1000
10000
0
5
10
15
20
25

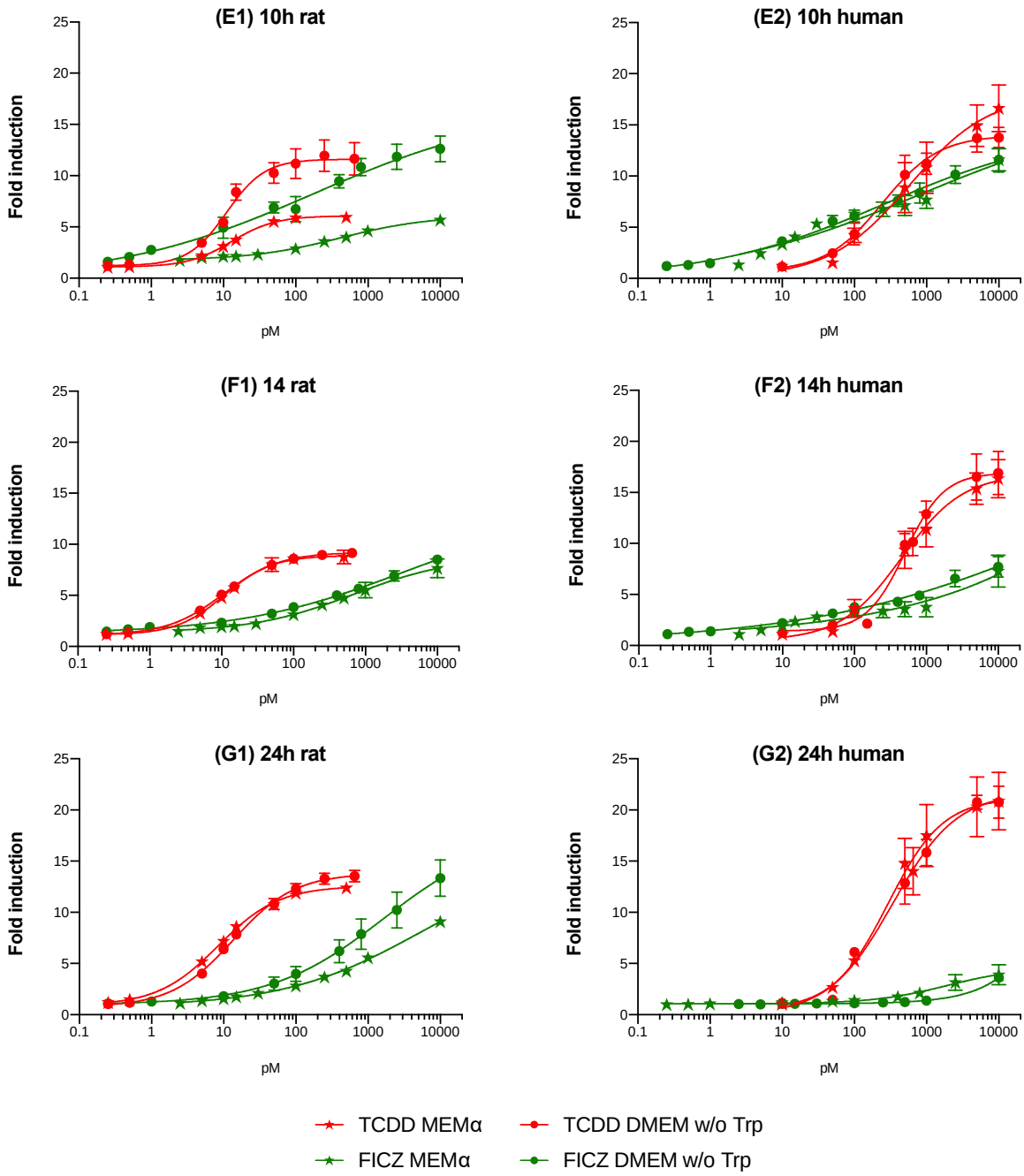


Figure 1. Dose response curves of rat DR-H4IIE and human DR-HepG2 cells exposed to TCDD or FICZ in culture medium with (MEMα) and without Trp (DMEM w/o Trp) from 2h to 24h induction.

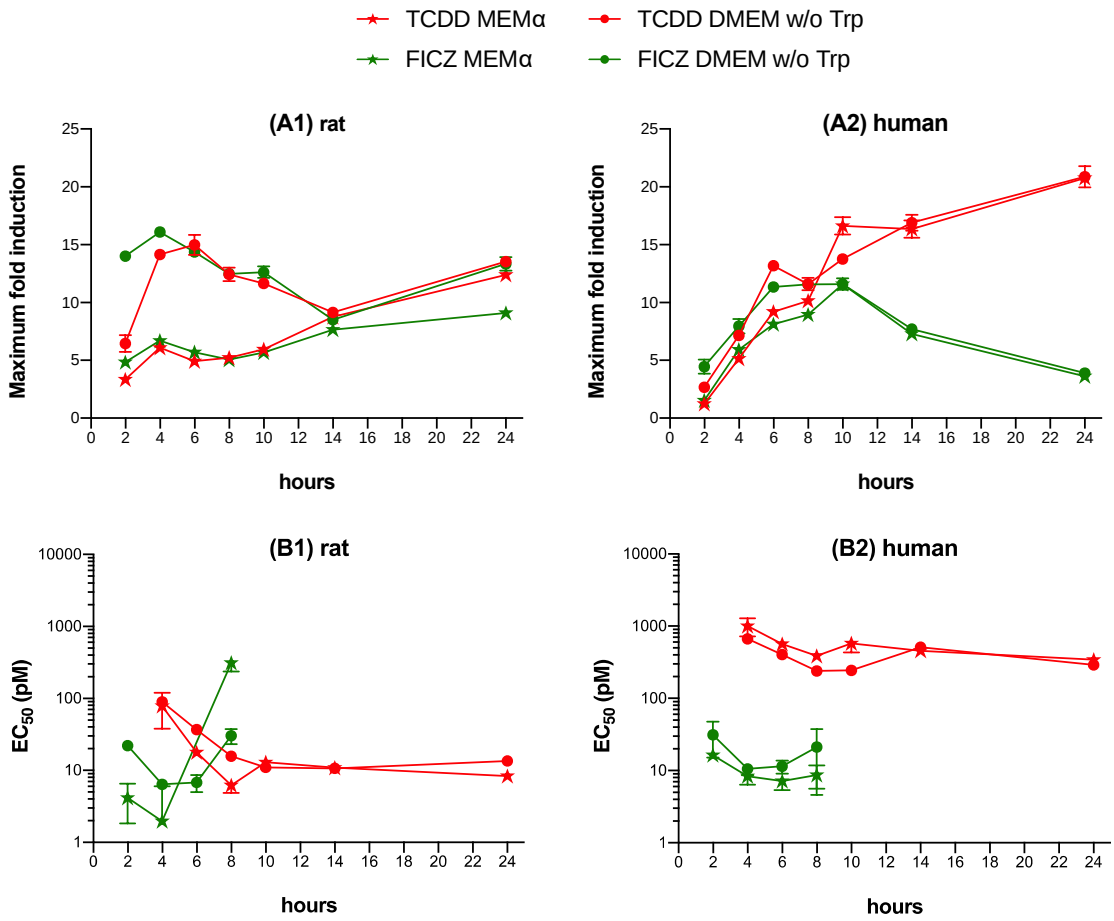


Figure 2. (A) Maximum fold induction for rat DR-H4IIE and human DR-HepG2 cells exposed to TCDD or FICZ in culture medium with (MEMα) and without Trp (DMEM w/o Trp) from 2h to 24h induction. (B) The respective EC_{50} values, only EC_{50} values of TCDD after 4h induction and EC_{50} values of FICZ before 10h induction were considered since their appropriate sigma dose response curves could be attained.

3.2. Rat and human aryl hydrocarbon receptor transcriptional activities of the polyphenols and the mixture

The seven individual polyphenols and their mixture were tested for AhR transcriptional activities in DR-H4IIE and DR-HepG2 in four different culture medial conditions (DMEM w/o Trp, MEMα, and in DMEM w/o Trp added either FICZ or TCDD EC_{50}). Two exposure times (4h and 8h) resulting in relatively stable AhR responses to FICZ and TCDD (section 3.1) were chosen for this experiment in order to avoid the complication of FICZ degradation through the metabolic feedback loop. The spike-in TCDD EC_{50} for 4h and 8h exposure were, respectively 85 pM and 15 pM in the rat DR-H4IIE, and 650 pM and 250 pM in the human DR-HepG2, while for FICZ, EC_{50} were, respectively 10 pM and 30 pM for both cell lines.

The polyphenols and mixture induced no cytotoxicity in both cell lines, except curcumin at 50 μM in the rat cells, which was excluded from the data presented. Their activity increased from 4h to 8h post-exposure and vanished after 24h (only tested in MEMα) in both cell lines, except chrysin which retained a moderate AhR activation after 24h exposure in the human cells with FI_{max} = 3.4 ± 0.5 fold (data not shown).

Daidzein and genistein, the two isoflavones, agonized the rat AhR significantly. Daidzein exerted FI_{max} = 4.7 ± 0.4 fold after 8h exposure in DMEM w/o Trp (Figure 3A1), compared to the 12-fold maximal induction by TCDD in the condition. In the co-exposure experiment, daidzein surprisingly boosted the rat AhR responses to both FICZ or TCDD EC_{50}, remarkably increasing the cells' response to FICZ EC_{50} up to maximum relative response RR_{max} = 744 ± 63% and that to TCDD EC_{50} up to RR_{max} = 361 ± 60% after 8h exposure (Figure 3A2). This resulted in a final 18 ± 3 and 9 ± 2 fold induction, respectively for FICZ and TCDD cotreatment after the spike-in EC_{50} reference subtraction, showing an almost 4 and 2-fold increase, compared to its respective agonistic activity alone.

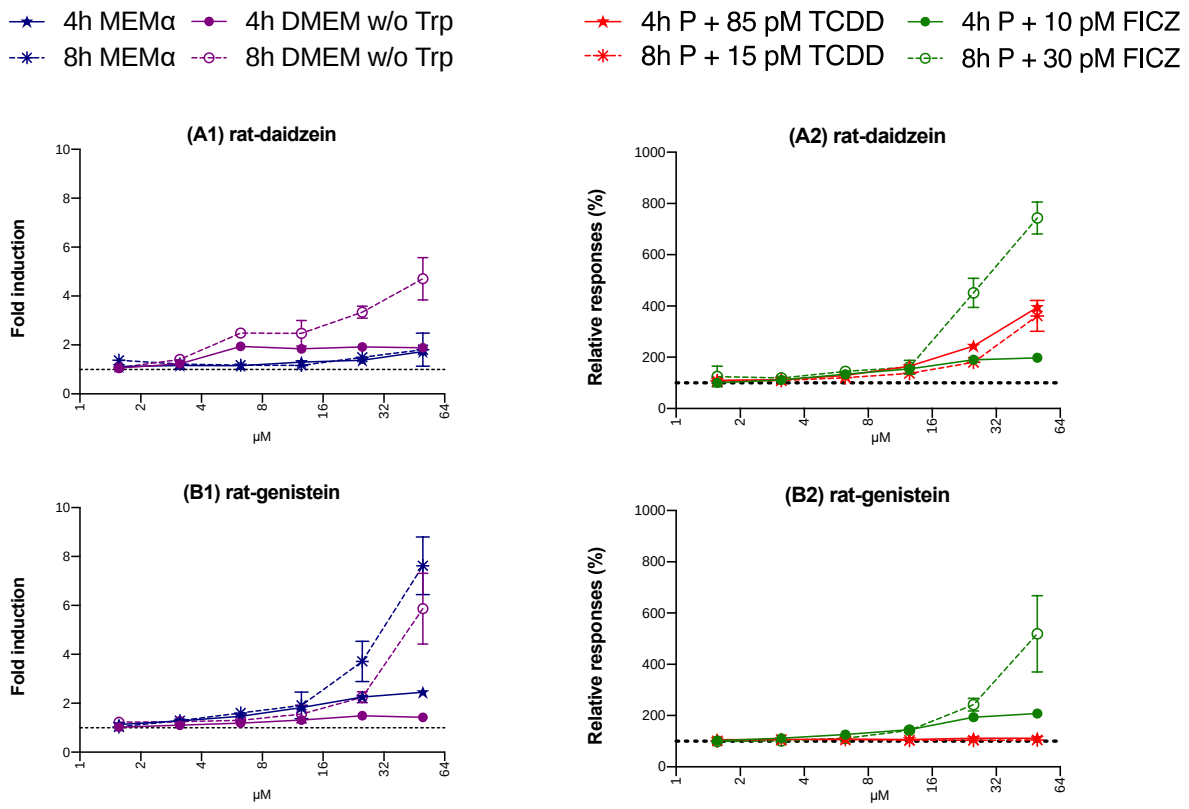


Figure 3. Dose response curves of rat DR-H4IIE cells responding to (A) daidzein or (B) genistein in (1) medium with (MEMα) or without Trp (DMEM w/o Trp) or (2) cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 4h or 8h induction.

Genistein stimulated the rat AhR in both MEMα and DMEM w/o Trp after 8h exposure with FI_{max} = 7 fold (Figure 3B1). Genistein also exerted an enhanced effect when co-exposing only with FICZ EC_{50} and only in rat cells after 8h exposure (RR_{max} = 520 ± 149%) (Figure 3B2), which resulted in a final 24 ± 3 fold induction, showing a 1.7-fold increase compared to its agonistic activity alone. This mutual enhancement of the response suggests the presence of a synergistic effect of the isoflavones with FICZ/TCDD, resulting in several fold increases in the induction efficiency when co-exposing with FICZ/TCDD.

Meanwhile, neither isoflavone displayed significant activities on the human AhR in DR-HepG2 (Figure S1). Noticeably, daidzein but not genistein antagonized TCDD EC_{50} in the human cells (RR_{max} = ~40%), but slightly increased the cells' response to FICZ EC_{50} (Figure S1A2), indicating a selective AhR inhibitory effect of daidzein on TCDD induction in the human cells.

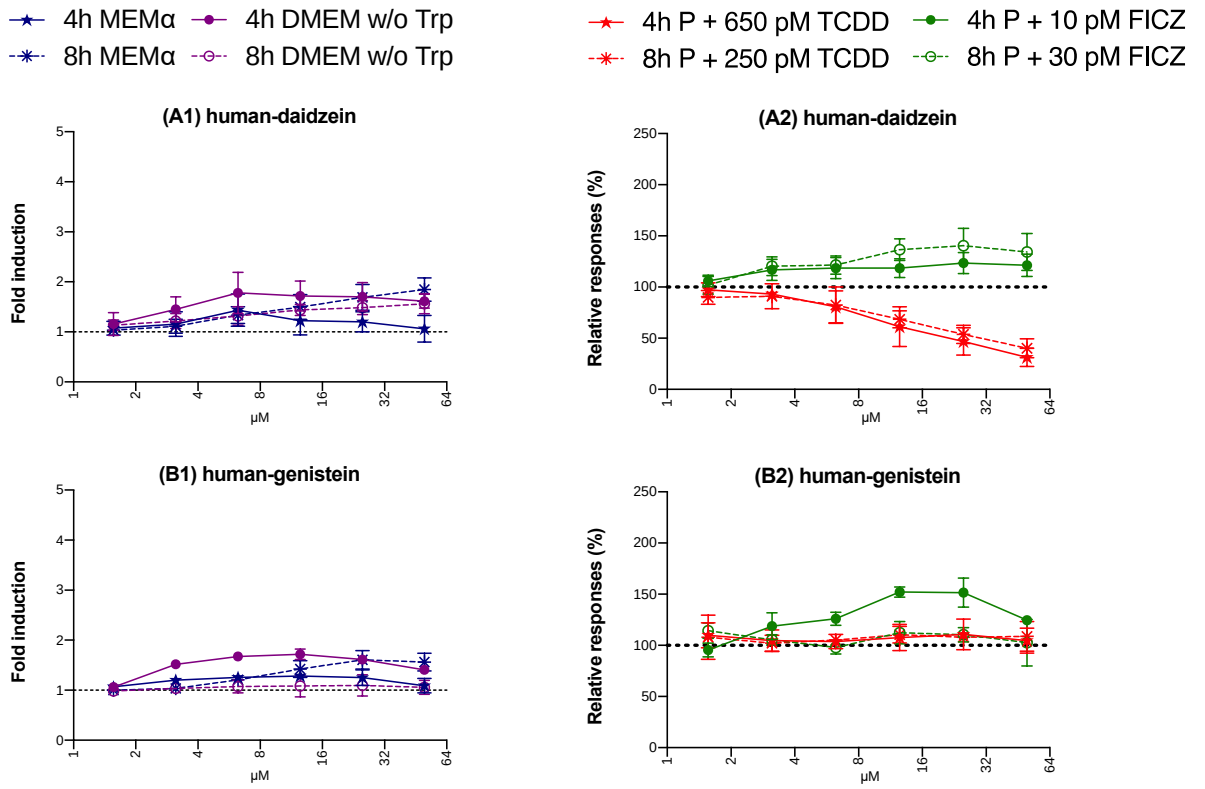


Figure S1. Dose response curves of human DR-HepG2 cells responding to (A) daidzein or (B) genistein in (1) medium with (MEMα) or without Trp (DMEM w/o Trp) or (2) cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 4h or 8h induction

In contrast to what was observed with the two isoflavones, flavonol quercetin, flavones (baicalin and chrysin), and curcumin affected the AhR transcriptional activity more strongly in human DR-HepG2 than in rat DR-H4IIE. In rat cells, they induced a weak AhR activation of

~2 fold only in MEMα culture medium (containing Trp) at their highest concentrations (Figure S2). In human cells, quercetin, baicalin, and chrysin exerted an agonistic effect regardless of the presence of Trp in the culture medium with FI_{max} = 3.2 ± 0.2 (Figure 4A1), 3.7 ± 0.1 (Figure 4B1), 5.2 ± 0.2 fold (Figure 4C1), respectively after 8h exposure in DMEM w/o Trp, compared to respective FI_{max} = 12 fold of TCDD. A similar response pattern was observed when MEMα was used as exposure medium. However, we observed a decrease in the response to the highest concentration (50 μM) of quercetin (Figure 4A1) and baicalin (Figure 4B1) after 4h exposure in DMEM w/o Trp, and chrysin (Figure 4C1) after 4h and 8h exposure in MEMα and 4h in DMEM w/o Trp.

On the other hand, only quercetin inhibited rat AhR activation by TCDD EC_{50} with RR_{max} = 33 ± 8% after 8h exposure, but the other two flavones did not (Figures S2A2; B2; and C2). In human cells, quercetin was the strongest AhR antagonist, followed by the two flavones. Quercetin at 3 μM totally abolished the activity of 30 pM FICZ (RR_{max} = 5 ± 3%) (Figure 4A2), while 15.5 μM of chrysin was needed for the same effect (RR_{max} = 9 ± 5%) (Figure 4B2), regardless of exposure time. Baicalin also caused an antagonistic effect but to a lesser extent (RR_{max} = 46 ± 7.5% at 50 μM) after 8h exposure (Figure 4C2). Both flavones did not affect the human AhR response to TCDD EC_{50}, while quercetin antagonized strongly TCDD after 4h and 8h exposure, in presence of respectively 650 pM and 250 pM TCDD, showing a V-shaped response curve (Figure 4A2). The lowest tested concentration of quercetin (0.3 μM) already reduced by half the TCDD EC_{50}-induced AhR response of the human cells after 8h exposure or completely after 4h exposure, while quercetin with 3 μM totally ablated the AhR activity of TCDD EC_{50} at both exposing times. The relative response increased to around RR_{max} = 50% at higher concentrations, probably reflecting the agonistic activity of quercetin at these concentrations.

Curcumin is a pure AhR inhibitor in the human cells, but not in the rat cells. In human cells, it antagonizes even the DMSO baseline of the human AhR response (Figure 4D1), and both TCDD EC_{50} and FICZ EC_{50} regardless of exposure time (Figure 4D2).

Resveratrol induced relatively similar AhR agonistic responses in both rat and human cells (Figures 5A1 and C1). Similar to the two isoflavones, resveratrol seems to have a synergistic activity with FICZ EC_{50} in the rat cells after 8h exposure (Figures 5A2). Meanwhile, in the human cells, similar to quercetin, only 6.3 μM resveratrol strongly antagonized TCDD EC_{50} to RR_{max} = 25 ± 7% after 8h induction in DMEM w/o Trp but without a distinct V-shaped dose response curve (Figure 5C2).

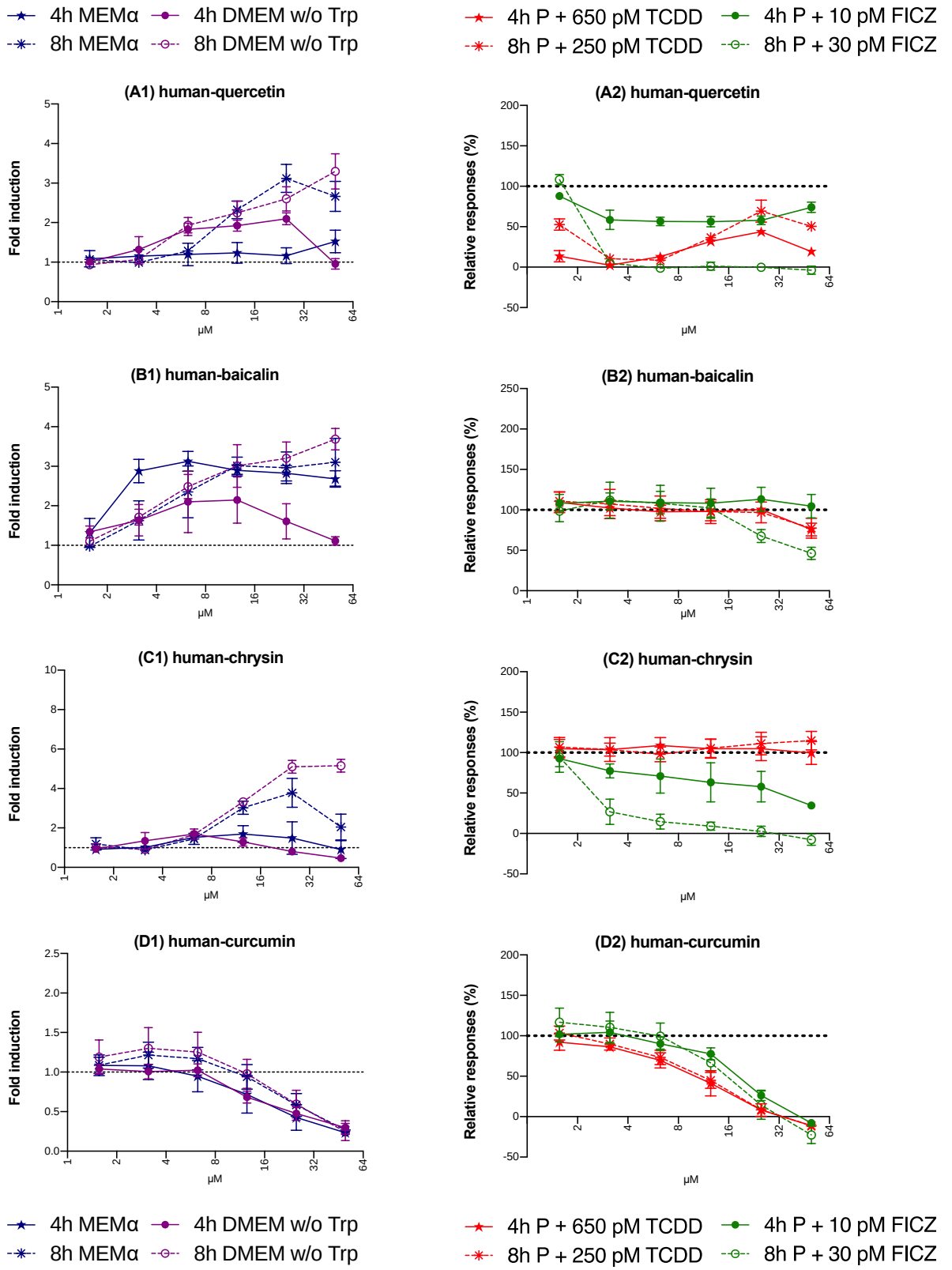


Figure 4. Dose response curves of human DR-HepG2 cells responding to (A) quercetin, (B) baicalin, (C) chrysin, or (D) curcumin in (1) medium with (MEMα) or without Trp (DMEM w/o Trp) or (2) cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 4h or 8h induction.

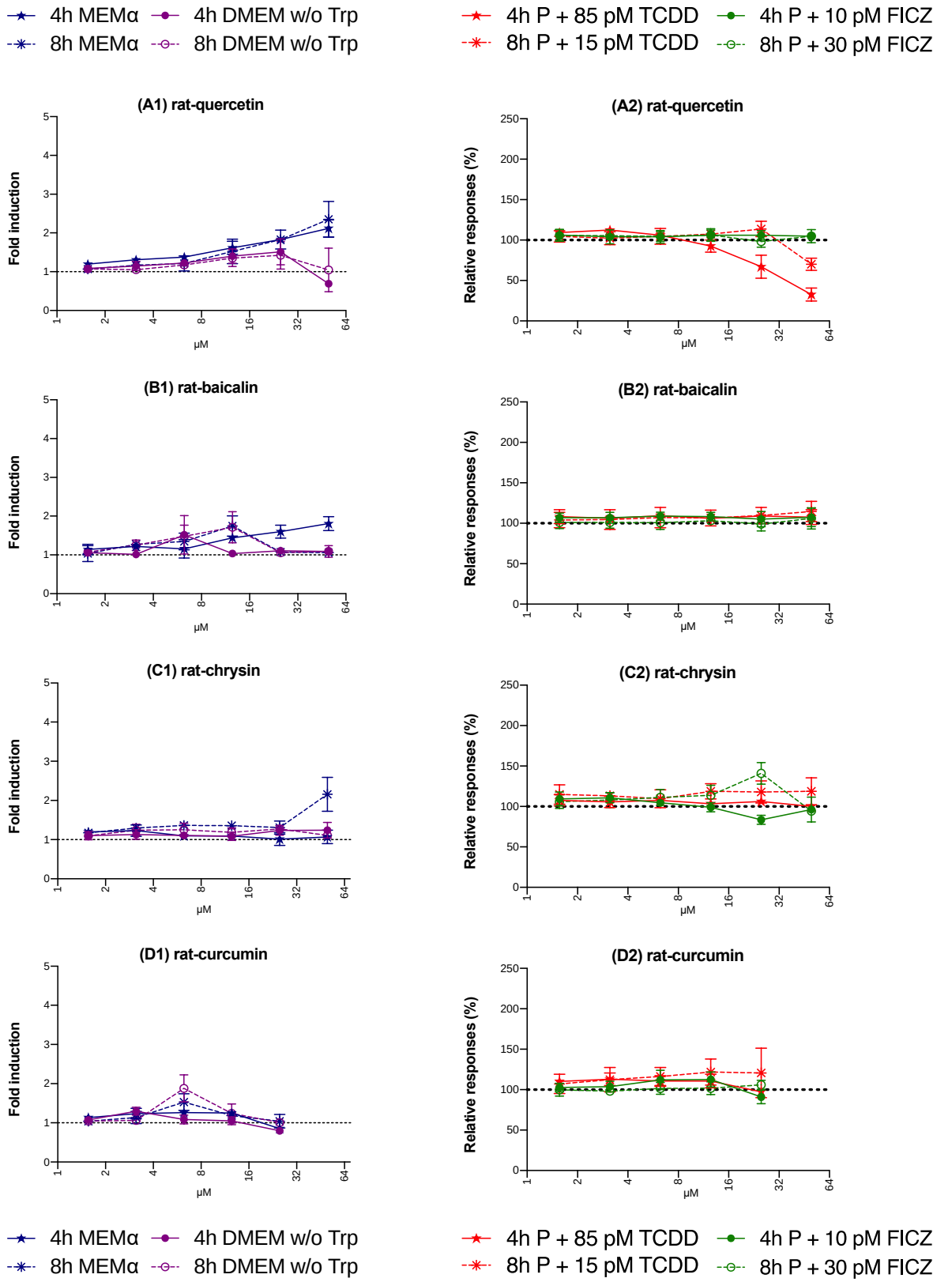


Figure S2. Dose response curves of rat DR-H4IIE cells responding to (A) quercetin, (B) baicalin, (C) chrysin, or (D) curcumin in (1) medium with (MEMα) or without Trp (DMEM w/o Trp) or (2) cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 4h or 8h induction

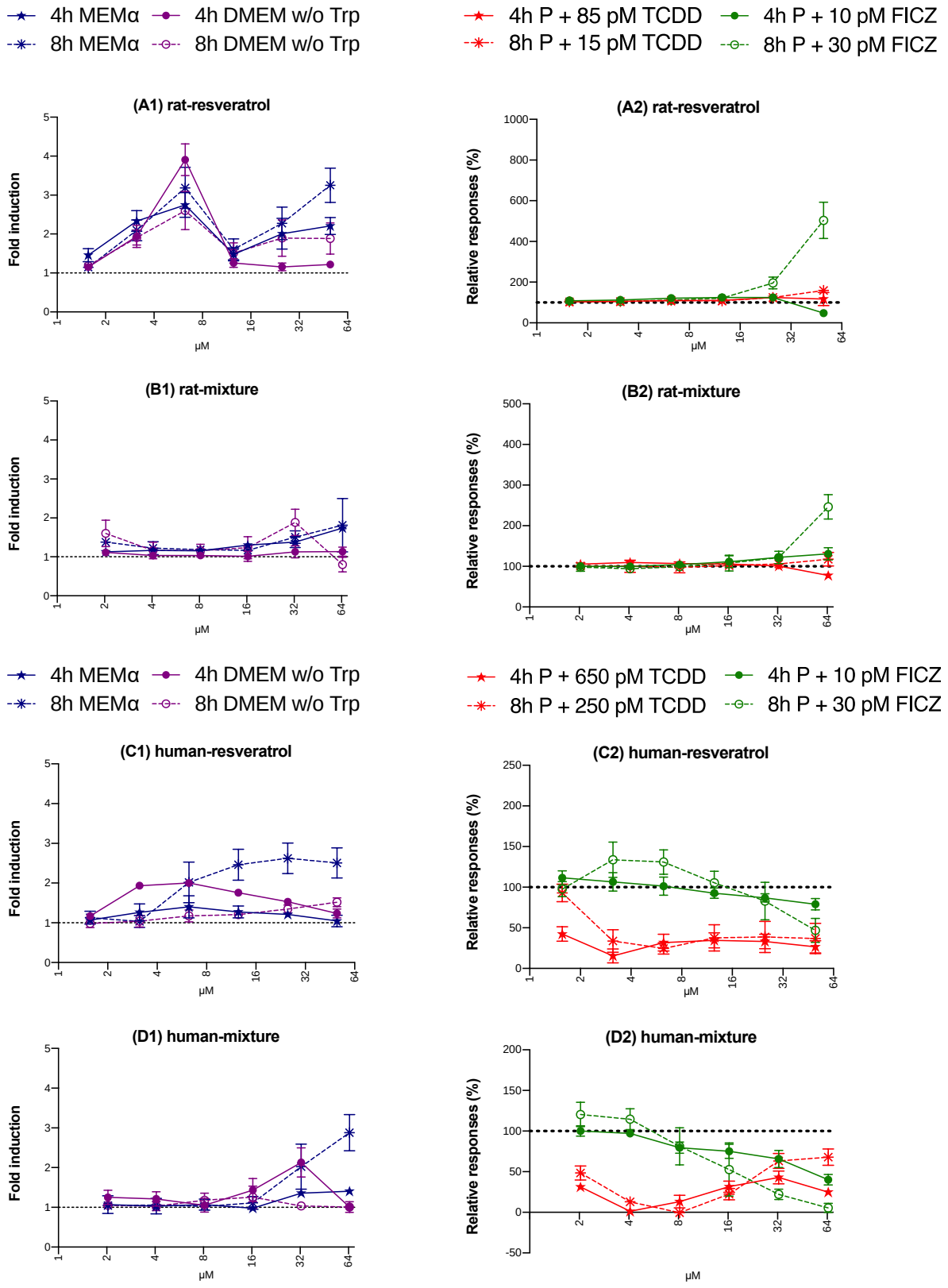


Figure 5: Dose response curves of rat DR-H4IIE cells responding to (A) resveratrol or (B) the mixture and of human DR-HepG2 cells responding to (C) resveratrol or (D) the mixture in (1) medium with (MEMα) or without Trp (DMEM w/o Trp) or (2) cotreated with FICZ or TCDD EC_{50} in DMEM w/o Trp for 4h or 8h induction.

The polyphenol mixture induced a weak AhR activation response relatively similar in both human and rat cells (Figures 5B1 and D1). However, while the mixture increased the rat AhR activation by FICZ EC_{50} after 8h exposure (Figure 5B2), as did the two isoflavones and resveratrol, it strongly antagonized both FICZ and TCDD EC_{50} AhR activation in the human cells with a dose-response curve reminiscent of that seen with quercetin (Figure 5D2). Only 4 μM of the mixture totally abolished the activity of 650 pM TCDD (RR_{max} = 1 ± 2% for 4h or even RR_{max} = 0 ± 2% for 8h in DMEM w/o Trp). The results are summarized in Table 1.

Table 1. AhR activities of the seven polyphenols. Activation (↑, orange), inhibition (↓, green) and no effect (grey).

Polyphenols	Cell species	DMEM w/o Trp	MEMα	DMEM w/o Trp + FICZ EC_{50}	DMEM w/o Trp + TCDD EC_{50}
Daidzein	Rat	↑		↑	↑
	Human				↓
Genistein	Rat	↑	↑	↑	
	Human			↑	
Quercetin	Rat		↑		↓
	Human	↑	↑	↓	↓
Baicalin	Rat				
	Human	↑	↑	↓	
Chrysin	Rat		↑		
	Human	↑	↑	↓	
Curcumin	Rat				
	Human	↓	↓	↓	↓
Resveratrol	Rat	↑	↑	↑	↓
	Human	↑	↑		
Mixture	Rat	↑	↑	↑	
	Human	↑	↑	↓	↓

3.3. Human aryl hydrocarbon receptor inhibitory activity of resveratrol, quercetin and the mixture

The TCDD inhibitory potentials in the human cells of resveratrol, quercetin, and the mixture at low concentrations were further tested in DMEM w/o Trp for 8h exposure. Figure 6A shows that the two polyphenols and the mixture exhibited a strong inhibitory effect against the human AhR activated by TCDD EC_{50}. While 5 μM resveratrol inhibited the AhR activity induced by TCDD EC_{50} (650 pM) to RR_{max} = ~20%, 2.5 μM quercetin or the mixture at blood concentration

(≅ 2.5 μM) was able to totally abolish this activity. Their TCDD inhibitory effects started at concentrations as low as 0.2 to 0.3 μM (Figure 6A), and their IC_{50} were 1 ± 0.08, 0.6 ± 0.03, and 0.8 ± 0.06 μM, respectively, for resveratrol, quercetin, and the mixture. Co-exposing these polyphenols at the same concentration (5μM) or the mixture with various concentrations of TCDD (0.15 to 20 nM) (Figure 6B) revealed that resveratrol was less efficient (a reduction of only 30% of TCDD 20 nM), while the mixture and quercetin blocked totally TCDD-induced AhR activation even the highest (20 nM) concentration.

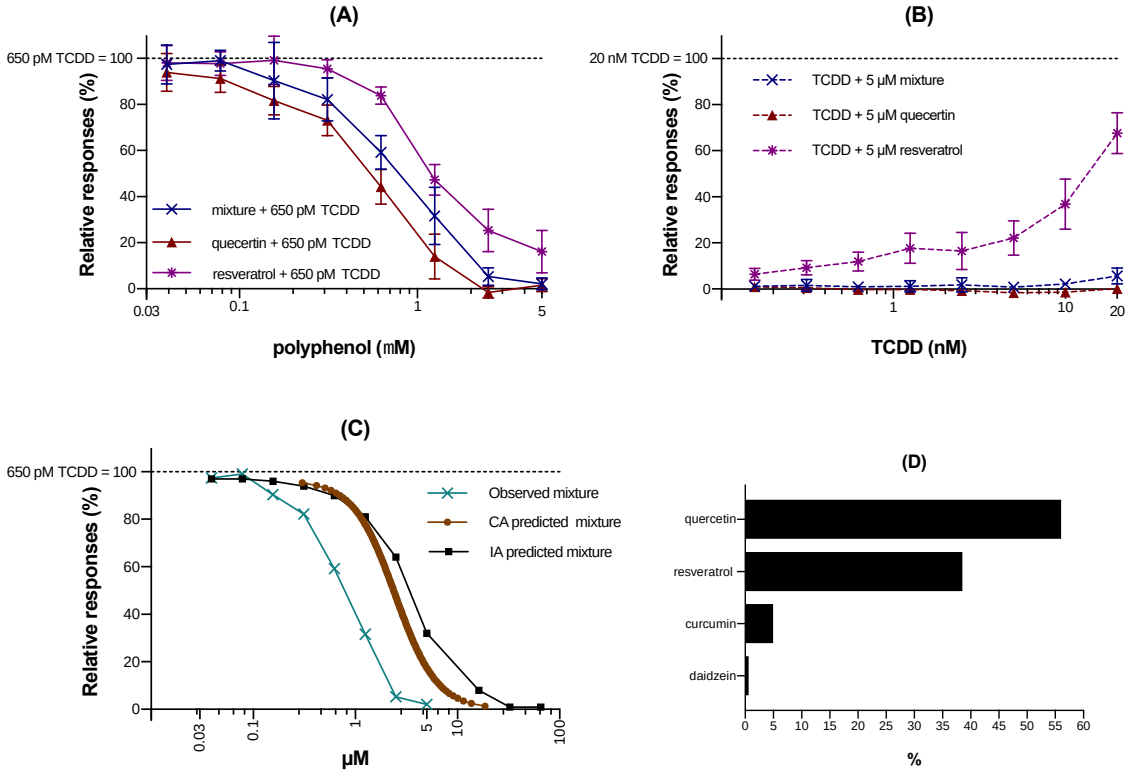


Figure 6: Dose response curves of DR-HepG2 cells responding to (A) the mixture, quercetin and resveratrol co-exposed with 650 pM TCDD, (B) various TCDD concentrations co-exposed with 5 μM of these polyphenols, (C) the predicted and measured dose response curve of the mixture after 8h induction, and (D) distribution of effect units of the 4 active AhR antagonists in the mixture.

The inhibitory effect on TCDD EC_{50} of the mixture in the human cells at 8h was predicted using concentration addition (CA) and independent action (IA) models[32] (Figure 6C) using the information of the four AhR antagonists namely, daidzein, quercetin, curcumin, and resveratrol. Thus, the human AhR elevated activation of the mixture observed at concentrations higher than 5 μM was not considered. Detailed calculations are presented in supplementary information “Calculations of mixture effect and effect unit”. Both the CA and IA models predicted a dose-response curve which closely overlapped the experimentally

measured curve. The IA model predicted an IC_{50}= 3.5 μM, close to 2.5 μM of the CA model, compared to 0.8 μM measured. Quercetin contributed for 56% of the effect of the mixture, while 39% was contributed by resveratrol (Figure 6D).

4. Discussion

4.1. Rat and human aryl hydrocarbon receptor responses to FICZ and TCDD

This study is the first to show a direct and cross-species (rat and human) comparison of AhR transactivation by FICZ and TCDD in hepatoma cells. We found that FICZ potency was similar on both rat and human AhR. However, FICZ was 40-fold more potent than TCDD in human cells (Figures 1 and 2). In a mouse knock-in model where the endogenous mouse *Ahr* gene was entirely replaced with the human *AHR* gene, a reduction of TCDD-induced *CYP1A* family genes expression was observed[27]. This is in agreement with our results showing that the human AhR has a lower affinity for TCDD than the rat AhR[32]. There is currently no data available for interspecies (rat and human) comparisons of AhR responses to FICZ. However, in an intraspecies comparison, the low affinity murine isoform (AhR_d) which is the result of an Alanine to Valine change at residue 375, showed a four-fold lower binding affinity for TCDD than the AhR_b high affinity variant[33]. Nevertheless, both variants exhibited similar sensitivity to FICZ. Also, FICZ functioned similarly between the two mouse strains, exerting no differences in the induction of T helper 17 immune cells[24]. Additionally, the binding of different ligands (*e.g.* FICZ versus TCDD) has been suggested to cause different AhR conformational changes, resulting in different downstream consequences[34], such as in modulating the recruitment of various coactivators and transcription factors[35]. For example, a comparison of TCDD and FICZ in an *in vivo* mouse model for infection by the influenza virus revealed that they differentially affected various cell-specific AhR activators, most likely due to the different conformational changes that they induced[36].

In this study, depletion of Trp in the medium (DMEM w/o Trp) did not affect the AhR responses in human cells, but led to an almost 3-fold higher induction by both TCDD and FICZ after 4h to 10h treatment in rat cells (Figure 2A1). A “pure” AhR antagonist, GNF315[37] at 10 μM inhibited half (reduced 64%) of the basal luciferase signal of the rat DR-H4IIE in MEMα after 6h treatment, but it had no significant effect (reduced only 16%) on the basal response in DR-HepG2 (data not shown). Thus, it is likely that on top of the activities of the FICZ added, the rat AhR responded to the high level of basal AhR activation caused by endogenously produced ligands derived from Trp upon addition of fresh medium. These ligands were unstable, as the difference in medium with and without Trp tended to vanish after 12h treatment. In human cells, the AhR transactivation of both TCDD and FICZ was not affected by the presence of Trp

in culture medium (Figure 2A2). This could result from a lower conversion rate of Trp to AhR endogenous ligands, or conversion to different compounds in human cells.

Moreover, we showed that FICZ clearance was high in the human cells. It appears that if FICZ clearance is blocked, such as by inhibiting the FICZ/AhR/CYP1A1 feedback loop, FICZ could also induce toxicity. In an *in vivo* research on zebrafish, Wincent *et al*., (2017)[38] showed a toxic effect of FICZ after knockdown of the *cyp1a1* gene, while a combined knockdown of *cyp1a1* and *ahr2* (homologous to *AhR* in humans) significantly reduced this toxicity. The authors concluded that the toxicity of FICZ was at least partially Ahr2-dependent in zebrafish embryos, similar to that of TCDD, but observed only when its degradation by Cyp1a1 is blocked.

Thus, it is at present difficult to pinpoint the reason for the ligand-intrinsic differences in AhR responses while different susceptibility to metabolism complicates the issue. However, the conservation in high FICZ potency in inducing AhR activation across different species and among different AhR isoforms suggests its physiological importance as an endogenous AhR ligand during evolution[16].

4.2. Rat and human aryl hydrocarbon receptor responses to the polyphenols and the mixture

This study, for the first time, reports the species-specific AhR transcriptional activity in cells exposed to different polyphenols. Isoflavones (daidzein and genistein) were more active in the rat DR-H4IIE, while the flavonol quercetin, flavones (baicalin and chrysin) and curcumin strongly affected the AhR activity in the human DR-HepG2. The stilbene resveratrol acted equally on both cell lines. Although further investigations involving larger numbers of polyphenols are necessary to evaluate the AhR species-responses of different polyphenol subclasses, the data are valuable for selecting polyphenols as active compounds specifically for human-aimed research and also raise a concern for traditional rodent-to-human extrapolation in risk assessments. It is important to stress here that these activities were only observed after short term exposures (4h or 8h), only chrysin maintained some of its activity after 24h.

The two isoflavones exerted AhR agonistic effects in the rat cells and dramatically enhanced the rat AhR response in the presence of FICZ EC_{50} (4 and 3.5-fold higher than acting alone for daidzein and genistein, respectively). Several previous studies revealed stronger FICZ induction after 24 or 48h by interfering with the clearance of FICZ via the FICZ/AhR/CYP1A1 feedback loop [39,40], however we chose to perform our experiments at 4 and 8 h post-exposure,

before degradation kicks in, therefore we most likely can rule out this mechanism for the observed synergistic effects. An enhanced induction was previously observed for the glucocorticoid receptor-mediated transcription due to sodium arsenite-induced chemical stress, or for progesterone or androgen stimulation by a combined treatment with forskolin (an activator of adenylate cyclase) and trichostatin A (an inhibitor of histone deacetylases)[41]. Likewise, a rat AhR synergistic activity of genistein with 300 pM TCDD was observed previously that was explained by the various cellular functions of genistein[29]. Thus, in our case, the synergistic effects of daidzein and genistein co-exposing with FICZ EC_{50} were likely due to their activity on other pathway(s) which helped to enhance AhR transactivation more efficiently.

On the other hand, these isoflavones did not affect FICZ-induced AhR activation in the human cells. Instead, daidzein, but not genistein strongly inhibited the TCDD-activated AhR transcription in the human cells (Figure S1 A2), which is in agreement with the finding that daidzein inhibited the transcription of *Cyp1* genes induced by a xenobiotic 7,12-dimethylbenz(a)anthracene[39].

The flavonol quercetin and the two flavones (baicalin and chrysin) exerted significant AhR agonistic and antagonistic effects in the human cells cultivated in media with or without Trp (Figures 4A1; B1; and C1) but not in the rat cells. Several mechanisms could explain the AhR agonistic activity of quercetin. Jin *et al*., (2018)[42] reported that quercetin was an inducer of CYP1A1 in CaCo-2 cells, and they also showed the interaction of quercetin with AhR using *in silico* tools. In addition, quercetin was shown to significantly increase the AhR translocation by activating protein kinases C in rats, facilitating the transcriptional activity of AhR in osteoblasts[43]. Phosphorylation by *e.g.* protein kinases C is required for AhR activity in DNA binding and gene transactivation and for the transformation of the unliganded AhR into a fully functionally active AhR/ARNT heterodimer[44].

AhR antagonism of quercetin and chrysin at concentrations higher than 5 μM was previously shown for DR-HepG2 after 6h exposure[29]. However, another original finding of this study is the strong AhR antagonistic activity of quercetin at very low concentrations (≤ 5 μM) in the human DR-HepG2 cells co-exposed to quercetin and increasing concentrations of TCDD (Figures 6A and B). One could suggest that quercetin was a TCDD competitive antagonist, strongly competing to TCDD for binding sites in the human AhR. However, the agonistic activity of quercetin in human cells was very low or absent at concentrations < 5μM, indicating its low AhR affinity (Figure 4A1). These observations indicate that quercetin may not be a

TCDD competitive antagonist. For example, quercetin was found to induce AhRR (AhR repressor) mRNA expression in human Caco-2 cells, thereby facilitating AhRR-dependent transrepression of AhR and inhibiting the BaP-AhR activation[45]. However, there could be additional underlaying mechanism(s) for the strong antagonistic effect of quercetin in the human DR-HepG2, which deserves further investigations.

Resveratrol was an AhR agonist in both rat and human cells. It also enhanced the response to FICZ of the rat AhR in the same manner as the two isoflavones did. It was also a strong AhR antagonist in the human cells, although less potent than quercetin (Figures 6A and B). Lee and Safe (2000)[46] showed that resveratrol did not exhibit AhR agonistic or antagonistic activity in human mammary carcinomaT47D or MCF-7 cells. Instead, it inhibited *CYP1A1* mRNA expression and CYP1A1-dependent activity via an AhR-independent post-transcriptional pathway. It promoted the nuclear translocation and/or DNA-binding activity of AhR, showing partial AhR-agonistic features. On the other hand, it reduced AhR-inducible gene expression without interfering with AhR-DRE binding, displaying its AhR-antagonistic effect when cells were co-exposed to TCDD. Similarly, another study showed that resveratrol provoked the translocation and binding of the AhR to DREs as well as competed with and displaced TCDD from AhR binding sites in T-47D and HepG2 cells, but without the subsequent transactivation of several dioxin-inducible genes including *CYP1A1*[47]. Likewise, it inhibited *in vivo* rat testicular CYP1A1 expression by decreasing the BaP-induced AhR protein level, AhR nuclear translocation and subsequent promoter activation[48]. Meanwhile, Perdew *et al.* (2010)[49] showed that CYP1A1 repression by resveratrol was dependent on estrogen receptor (ER) since siRNA against ERα was able to completely abolish the repressive effect of resveratrol on TCDD-inducible CYP1A1 mRNA accumulation in MCF-7 cells, but not in Caco-2 cells, indicating cell-specificity.

We showed that curcumin exerted an AhR antagonistic activity only in the human DR-HepG2 cells. This activity is probably due to the inhibition of TCDD-induced AhR phosphorylation. Nishiumi *et al.* (2007)[50] showed that in mouse Hepa-1c1c7 cells, curcumin accelerated the TCDD-induced AhR translocation, but inhibited heterodimerization of the AhR with ARNT by dose-dependently inhibiting the TCDD-induced phosphorylation of both the AhR and ARNT, thereby suppressing AhR activation. Furthermore, Cho *et al.* (2008)[51] revealed the involvement of oxidative stress in mediating the curcumin-induced degradation of AhR and ARNT. Similarly, Nakai *et al.* (2018)[52] found that curcumin suppressed TCDD-induced protein expression and activity of CYP1A1 in human HepG2 cells and mouse Hepa-1c1c7 cells by inhibiting phosphorylation of AhR. These mechanisms might be less involved, or carried out by different cell components in the rat DR-H4IIE cells[53], thus explaining the nearly complete

absence of AhR antagonism of curcumin in this study. To conclude, it seems that the effect of polyphenols on the AhR transcriptional activities is far more complicated and rarely solely as a direct AhR ligand, instead involving multi-cellular pathways.

The mixture of the seven polyphenols exerted an AhR agonistic activity in both rat and human cells. Interestingly, similarly to quercetin, it also inhibited strongly the AhR activation induced by TCDD at various concentrations in the human cells (Figure 6B). Both CA and IA models resulted in an acceptable prediction of the antagonistic mixture effect (Figure 6C), indicating that the activities of these polyphenols were additive in the mixture with a similar or dissimilar mode of actions.

Quercetin and resveratrol acted together, contributing to 95% of the AhR antagonism of the mixture, which was able to totally abolish the AhR activation of 650 pM TCDD in DR-HepG2 cells, at a concentration of 2.5 μM (Figure 6A). At this concentration, the mixture contained 0.33 μM quercetin and 0.44 μM resveratrol, corresponding to theoretical calculated blood levels (Table S1). According to literature, these concentrations could be realistic in human blood after dietary polyphenol ingestion. Quercetin is naturally found in onions, apples, broccoli, and berries[54,55], while resveratrol is abundant in red grapes, red wine, peanuts, and ground nuts[56]. Two main metabolites of quercetin, quercetin-30-sulphate and quercetin-3-glucuronide, accumulated in human plasma with maximum concentration of 0.665 μM and 0.351 μM respectively after less than an hour after the ingestion of 270 g fried onions[57]. Similarly, the blood concentration of resveratrol can peak at 2 μM after an hour of single administration of an oral dose of 25 mg of *trans*-resveratrol to healthy volunteers[58].

In our previous study[32], we showed that a mixture of 29 persistent organic pollutants, the total POP mixture, prevalent in Scandinavian human blood, also caused an AhR antagonistic effect, which could be realistic. While this total POP mixture can cause a persisting AhR antagonism, the AhR antagonistic effects of quercetin, resveratrol, and the mixture of polyphenols are only transient due to their fast metabolism. However, the health impact of the human exposure to these multiple chemicals responsible of AhR antagonism should be assessed.

Conclusion

We showed for the first time a side-by-side comparison of the effects of an endogenous AhR ligand (FICZ) and exogenous ligand (TCDD) on two hepatoma cell lines from two different organisms (rat and human). FICZ was 40 times more potent than TCDD in the human cells,

and displayed similar potency in both rat and human cells, indicating its physiological importance as an endogenous AhR ligand during evolution. Furthermore, we showed a huge effect of Trp depletion on AhR activation in rat cells, but none in human cells, probably due to the higher production of endogenous ligands from Trp in rat cells, along with a faster degradation of the ligand FICZ in human cells. The effect of seven polyphenols and their mixture thereof on AhR transactivation alone and after co-exposing with FICZ or TCDD was also investigated. Both agonistic and antagonistic effects were observed, in line with the expected complexity of a system where differential degradation of the tested compounds was certainly playing a role, as well as interactions of the compounds, and their metabolites, with various components of the cellular machinery. Antagonistic activity of quercetin, resveratrol, and the mixture towards TCDD-induced human AhR activation was observed in this study at nanomolar concentration, which is realistic in human exposure levels.

FICZ and other endogenous ligands together with the either agonistic or antagonistic activities of polyphenols, in concert, regulate and maintain the physiological functions of AhR in balance. This balance can be disrupted by xenobiotic exposure or mis-applications of natural supplements but also can be used to treat diseases related to AhR disfunctions. Further studies are required to fully understand the interference of these dietary polyphenols on the AhR mediated activity of both endogenous and persistent xenobiotic AhR ligands in order to assess the health impact of their use as drugs or food supplements.

Conflict of interest: The authors declare that there is no conflict of interest.

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Supplementary materials.

Calculations of mixture effect and effect unit. The predicted antagonistic mixture effect was calculated by using models of concentration addition (CA) and independent action (IA)[32]. CA model is applied for chemicals acting in the same mode of action, calculating the effect concentration ($IC_{mix,j}$) of the mixture inducing a specific antagonistic effect j (from 1% to 100%) by considering the concentration partition (p_i) of compound i and its respective effect concentration (IC_{ij}) inducing the same effect j (Eq. (S1)). Previously published formulae were adapted[59–61]:

$$(S1)\ \ IC_{mix,j} = \left(\sum_{i=1}^{n} \frac{p_i}{IC_{ij}}\right)^{-1}$$

For each compound i, IC_{ij} inducing the effect j is calculated using its $IC_{i,50}$ and hillslope (H_i) from their fitted curves using Eq. (S2) (Graphpad PRISM):

$$(S2)\ \ IC_{ij} = IC_{i,50}\left(\frac{j}{100-j}\right)^{1/H_i}$$

To generate the full dose-response curves, the hillslope and top (H_{mix} and T_{mix}) of the mixture response were calculated as the average responses of the active components (n) due to their similarity (Eq. (S3a and b)), while the bottom (B_{mix}) was calculated by Eq. (S3c) considering their weight (p_i) in the mixture:

$$(S3a) H_{mix} = \left(\sum_{i=1}^{n} p_i H_i\right)\frac{100}{n}$$

$$(S3b) T_{mix} = \left(\sum_{i=1}^{n} p_i T_i\right)\frac{100}{n}$$

$$(S3c)\ B_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{B_i}\right)^{-1}$$

Deriving from CA model, effect units (*i.e*. the ratio of the concentration partition of a compound to its IC_{50} ($p_i/IC_{50,i}$), scaling the concentrations of the mixture components to its effect), has been applied to identify the main driver(s) for mixture effects in CA model[59,62,63].

IA model is applied for chemicals acting in different mode of action[64]. An antagonistic effect induced by compound i is obtained by subtracting the measured relative response (R_{ik}) from

100% (100% being the relative response of TCDD EC_{50}) and then converted into a probability (scale 0-1, by dividing by 100). The relative response of the mixture (0%-100%) is calculated from the combination of individual probabilities of each compound using the adapted formula Eq. (S4)[60,62]:

$$(S4)\ R_{mix,k} = 1 - \left(1 - \prod_{i=1}^{n}\left(1 - \frac{100 - R_{ik}}{100}\right)\right)$$

At a specific concentration k, $R_{mix,k}$ is the relative response of the mixture; R_{ik} is the relative response of compound i at that concentration k of the mixture, n is the number of the active components.

Chapter 4

Access to safe drinking water is crucial for public health, however drinking water is also an unavoidable route exposing humans to pollutants, especially endocrine disruptors (EDs). Although there are several legislations imposing maximal limits of EDs in drinking water, there is still a need for research on protecting human health against the effects of EDs in drinking water, especially in mixture forms. Prioritizing chemicals posing threats is crucial, considering the cost of water treatment, remediation, and monitoring.

The chapter 4 focuses on profiling the potential endocrine disrupting activities of the 18 selected compounds prevalent in raw water intended for drinking water production in Wallonia found in the BIODIEN project ("search for EDs in waters") funded by the Walloon Government in Belgium. A panel of reporter gene cell lines, including aryl hydrocarbon receptor (AhR) and the steroid receptors was used. Both agonistic and antagonistic activities of these compounds and their mixtures were investigated by using CALUX bioassays with a panel of seven transgenic cell lines: three (human and rat) responsive to dioxins through AhR and four (human) responsive to steroids through estrogen (ER), androgen (AR), progesterone (PR), and glucocorticoid (GR) receptors. The aims were to (a) assess both transcriptional agonistic and antagonistic activities of the 18 compounds individually and in mixtures towards the five receptors, (b) identify the most concerning endocrine disrupting compound(s), and (c) identify the main compound(s) responsible for the observed effects of the mixtures.

The results obtained have been submitted to Chemosphere.

In vitro profiling of the potential endocrine disrupting activities affecting steroid and aryl hydrocarbon receptors of compounds and mixtures prevalent in human drinking water resources

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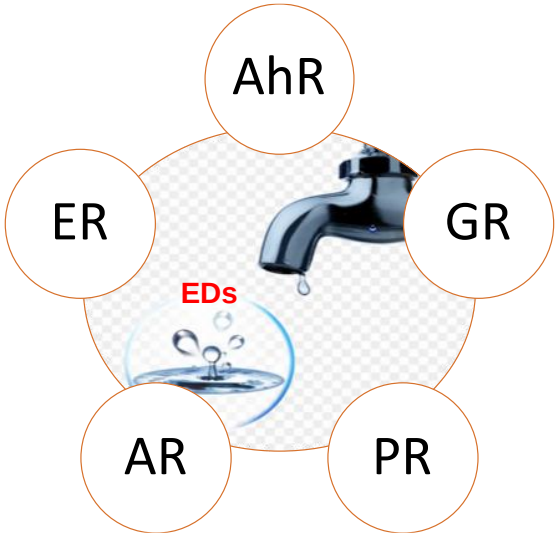
Abstract

Access to safe drinking water is crucial for public health since drinking water is an unavoidable route of exposing humans to pollutants. Prioritizing chemicals of concern for monitoring of drinking water contaminants is crucial for legislation when also considering costs of water treatment and remediation. We profiled *in vitro* potential endocrine disrupting activities of 18 compounds most frequently found in Walloon raw water intended for drinking water production. These compounds include pesticides, perfluorinated compounds, polycyclic aromatic hydrocarbons, and bisphenol A. Both agonistic and antagonistic activities of these compounds and their mixtures were investigated using a panel of CALUX bioassays incorporating seven reporter transgenic cell lines: three (human and rat) responsive to dioxins through the aryl hydrocarbon receptor (AhR) and four (human) responsive to steroids through the estrogen (ER), androgen (AR), progesterone (PR), and glucocorticoid (GR) receptors. Among them, ten caused at least one response in at least one receptor. Specifically, chlorpyrifos, bisphenol A, fluoranthene, phenanthrene, and benzo(a)pyrene displayed significant activities on several receptors. Bisphenol A agonized ER, but abolished the cells' response to androgen and progesterone. While fluoranthene and phenanthrene strongly reduced human AhR and AR transactivation, benzo(a)pyrene strongly activated AhR and ER, but inhibited GR and AR. In human breast cancer cells, benzo(a)pyrene dramatically activated AhR, inducing a 10-fold higher response than dioxin at concentrations realistically found in

blood from highly exposed sub-populations or via food contamination incidents. The mixture of the 18 compounds exerted both ER and rat AhR agonism, with the main contribution being benzo(a)pyrene or its combination with bisphenol A. Moreover, the mixture significantly inhibited dioxin-induced CYP1A activity (detected only by EROD assays) in human liver cells.

Keywords: Raw water; CALUX assays; Endocrine disrupting activities; Aryl hydrocarbon receptor; Steroid receptors; Benzo(a)pyrene.

Graphic abstract:



Highlights:

- Endocrine disrupting *in vitro* profile of 18 pollutants in drinking water resources.
- Chlorpyrifos, BPA, fluoranthene, phenanthrene, BaP display significant activities.
- BaP strongly induces AhR in human breast cancer cells, 10-fold higher than dioxin.
- The mixture exerts ER and rat AhR agonisms, due to BaP or its combination with BPA.
- EROD assays can detect effects of the mixture on human AhR-induced CYP1A activity.

1. Introduction

Since drinking water is an unavoidable route exposing humans to pollutants, access to safe drinking water is crucial for public health[1]. Endocrine disruptors (EDs), a group of exogenous substances that can interfere with the endocrine system, thereby affecting the health of animals and humans, are of particular concern. Exposure to EDs even at low doses is associated with several health problems including abnormal reproductive activity, high incidence of cancers, metabolic disorders such as obesity and cardiovascular diseases[2–4]. Several legislations impose maximal limits of EDs in drinking water. The Safe Drinking Water Act (SDWA) in the US[5], the Drinking Water Guidelines (ADWG) in Australia[6], and the Drinking Water Directive 98/83/EC in the European Union (EU)[7] have been enforced for the quality of water intended for human consumption.

While there is still a need for research on protecting human health against the effects of EDs in drinking water[4], especially in mixture forms, prioritizing chemicals posing the threats is crucial, considering the cost of water treatment, remediation, and monitoring. The Walloon Government in Belgium has recently funded the BIODIEN project ("search for EDs in waters") aiming to quantify the contamination by specific EDs of several Walloon and Brussels water bodies (surface and groundwater intended or not for drinking water production, waste water treatment plant effluents, runoff, *etc.*). Among the 194 compounds monitored, 58 have been quantified at least once in 100 samples of raw water (surface and ground) intended for drinking water production[8]. However, the health effects associated with these 58 chemicals have not yet been investigated, thus it is difficult to prioritize the regulation of certain compounds/groups.

Among methods evaluating the activity of EDs, the reporter gene "Chemically Activated LUciferase gene eXpression" (CALUX) cell-based assays have been introduced as a cost-effective, high-throughput-amenable and routinely applicable screening technique. They have been internationally validated to provide mechanistic and semi-quantitative data[9,10]. EDs can alter the endocrine system in various ways, one of them is by affecting the transcriptional activities of the steroid (estrogen (ER), androgen (AR), glucocorticoid (GR), and progesterone (PR)) receptors, and the aryl hydrocarbon receptor (AhR) which controls xenobiotic metabolism[4,11].

These four ligand-activated steroid receptors are regulated by steroids such as 17β-estradiol (E2), 5-α-dihydrotestosterone (DHT), dexamethasone (Dex), progesterone (P4), respectively, while 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the typical ligand of the AhR. The ligand-bound receptors bind to their respective cognate DNA sequences located in the promotor

regions of the target genes to activate their transcription[12,13]. CALUX assays take advantage of this series of events to establish reporter cell lines that are able to respond to the presence of ligands/stimuli[14]. Therefore, the assays accurately reproduce the mechanism of action of these compounds as in a living organism[15]. They are also able to discriminate between agonistic and antagonistic activities (by co-exposing the test compound with a typical agonist)[14]. Moreover, a good correlation has been reported between *in vitro* results of CALUX assays and *in vivo* experiments for several EDs[16,17]. Plus, the assays are able to reveal unknown properties and the complex response to mixtures[14].

In this study, the endocrine disrupting activities of 18 compounds most frequently found in Wallonia raw water intended for drinking water production and their mixtures were investigated using reporter gene assays for five receptors (AhR, ER, AR, GR, and PR). The aims were to (a) assess both transcriptional agonistic and antagonistic activities of the 18 compounds individually and in mixtures towards the five receptors, (b) identify the most concerning endocrine disrupting compound(s), and (c) identify the main compound(s) responsible for the observed effects of the mixtures.

2. Materials and methods

2.1. Chemicals and suppliers

We selected 19 compounds named from C1 to C19 based on their highest frequency of quantification (> 8%) in the raw water samples from the BIODIEN project. C4 (VIS01) was excluded as no analytical standard is available on the market. The main results of their monitoring during the BIODIEN project and their suppliers are given in Table 1. The 18 selected compounds belong to several groups: pesticides, perfluorinated compounds (PFCs), polycyclic aromatic hydrocarbons (PAHs), and a plasticizer. The largest share of contamination was due to nine pesticides and pesticide metabolites: C1 to C7, C14, and C15, with C1 contributing for 83% of their total mass. Five PFCs: C9 to C13 and three PAHs: C17 to C19, were also included with lower weight contribution, along with C8 (1H-benzotriazole), a versatile compound, and C16 (bisphenol A, BPA), a plasticizer. Reference standards, E2, DHT, Dex, and P4 were supplied by Sigma Aldrich (Missouri, USA), while TCDD was provided by Wellington (Campro Scientific, The Netherlands). All chemicals were dissolved in dimethylsulfoxide (DMSO) from Acros Organics, France, and stored at −20°C. Tested concentration ranged from 1.25 μM to 40 μM in a series of six 2-fold dilutions, which has been shown to be suitable for the CALUX assays without cytotoxicity[18,19]

Table 1. List of the 19 compounds most frequently found in raw water samples in the Wallonia and Brussels areas[8].

Name	Compound	CAS	Supplier reference	FOQ (%)	C_{max} (ng/L)	C_m (ng/L)	C_t (µM)
C1	Desphenyl-chloridazon (DPC)	6339-19-1	AKOS-006278170	82	21500	1230	8.5
C2	Methyl-desphenyl-chloridazon (Me-DPC)	17254-80-7	DRE-C11322500	69.7	1930	1320	8.3
C3	Desethylatrazine	6190-65-4	Sigma 36629	67.4	122	111	0.59
C4	VIS 01/Chlorothalonil SA	1418095-02-9	Not included	55.1	772	NA	NA
C5	Metolachlor ESA	171118-09-5	DRE-CA15171100	53.9	890	101	0.47
C6	Atrazine	1912-24-9	Sigma 45330	44.2	101	450	1.3
C7	Metazachlor ESA (BH479-8)	172960-62-2	DRE-CA14950020	40.4	186	130	0.4
C8	1H-benzotriazole	95-14-7	Sigma 76457	34.7	186	NA	1.6
C9	Perfluorooctanesulfonic acid (PFOS)	1763-23-1	Sigma 33829	29	19.95	NA	0.04
C10	Perfluorooctanoic acid (PFOA)	335-67-1	Sigma 33824	28	5.51	NA	0.01
C11	Perfluorohexanoic acid (PFHxA)	307-24-4	DRE-C15986910	23	11.53	NA	0.04
C12	Perfluoroheptanoic acid (PFHpA)	375-85-9	Sigma 342041	18	2.18	NA	0.01
C13	Perfluorohexanesulfonic acid (PFHxS)	355-46-4	Sigma 50929	13	15.02	NA	0.03
C14	Simazine	122-34-9	Sigma 32059	10.5	23	28	0.14
C15	Chlorpyrifos	2921-88-2	Sigma 45395	10.5	104	92	0.26
C16	Bisphenol A (BPA)	80-05-7	Sigma 239658	9.1	31	NA	0.14
C17	Fluoranthene	206-44-0	Sigma 45504	8.4	62	11	0.05
C18	Phenanthrene	85-01-8	Sigma 695114	8.4	19	29	0.16
C19	Benzo(a)pyrene (BaP)	50-32-8	Sigma B1760	8.4	70	29	0.12

FOQ: frequency of quantification (% of samples), C_{max}: maximum concentration quantified in raw water samples. C_m: measured concentration by LC-MS/MS and GC-MS/MS in the ED mixture. C_t: the maximum tested concentration in the ED mixture corresponding to 1,000 fold maximum water levels, except 100 fold for C1. NA: not applicable.

2.2. Mixture preparation

A mixture of the 18 chemicals called ED mixture, was constructed according to the maximum detected concentrations (C_{max}) in the raw water samples by pooling each component from their respective stock solutions. The concentrations of pesticides and PAH in the mixture were measured by liquid chromatography- tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) using in-house methods available in Société Wallonne des Eaux (SWDE) (see "measured concentration-C_m" in Table 1). PAHs were present in the mixture at lower concentration than expected, which could be due to their absorption by the glass container. To study the possible interactions among compound groups, a PFC mixture (C9-C13), a PEST mixture (C1-C8 and C14-C16), and a PAH (C17-C19) mixture were also prepared. The tested concentrations of the mixtures are given as "fold maximal water levels" relative to their concentrations present in water samples (Table 1). The maximum final tested concentration (C_t) was 1,000-fold, except for C1 which was 100 fold, due to its very high concentration measured in the water samples.

2.3. Transgenic cell lines

The transcriptional activity of these chemicals and the mixtures was investigated using seven genetically modified cell lines expressing the reporter gene luciferase. Three cell lines: rat hepatoma DR-H4IIE from BioDetection System (Amsterdam, The Netherlands), human hepatoma DR-HepG2 and human mammary gland DR-T47-D (both produced on site in Liege, Belgium)[20], were used to compare the AhR transcriptional activity between species (rat and human) and tissues (liver and mammary gland). DR-H4IIE cells were stably transformed with a vector containing four native dioxin responsive elements (DREs) from the upstream promotor of the mouse *Cyp1a1* gene with a MMTV (mouse mammary tumor virus) promoter[21], while four synthetic DREs regulating the thymidine kinase promoter drive luciferase expression in both DR-T47-D and DR-HepG2[20].

The four reporter cell lines addressed human steroid receptors, namely ER (ER-MMV), AR (TARM), PR (PR-TM), and GR (TGRM) as previously described[14]. ER-MMV cells were MCF-7 cells transformed with a vector containing the luciferase gene controlled by a vitellogenin promoter. The other three steroid reporter cells were derived from T47-D cells sharing a common vector containing the MMTV-LTR (long terminal repeat) promoter, inducible by glucocorticoid, progesterone, and androgen receptors[14]. While ER-MMV and PR-TM responded through their endogenous human receptors, TGRM and TARM were stably transformed with an additional expression vector coding for, respectively human GR and AR.

The cells were maintained in MEMα (ThermoFisher Scientific, Massachusetts, USA), except for ER-MMV in DMEM (ThermoFisher Scientific) supplemented with 1 µgm/L bovine pancreas insulin (Sigma Aldrich). These media were supplemented with 10% v/v fetal bovine serum (FBS) (Greiner, Kremsmünster, Austria), 50 IU/mL penicillin and 50 mg/mL streptomycin (Sigma Aldrich). The cells were incubated in a H_20 saturated atmosphere containing 5% CO_2, at 37_oC.

2.4. Reporter gene assays

Different protocols for each cell line were described in detail elsewhere[14,20]. Briefly, after 24h of cell seeding in white clear-bottomed 96 well microplates (Greiner), the medium was refreshed and the cells were exposed in triplicate with a dilution series of the individual tested compound or the mixture mentioned above. A reference curve with the reference ligand was performed on each plate for quality control. For co-exposure tests, the same dilution series was used on cells that were cotreated with a constant ~EC_{50} concentration of the reference compound (Table S1). The AhR-reporter cells were exposed for 24h in MEMα, while the steroids were exposed for 48h in DMEM without phenol red and supplemented with charcoal stripped FBS to eliminate natural steroid activators. After exposure, the cells were lysed by solutions containing Triton X100 (Sigma Aldrich). The luminescence signal was recorded by adding luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) to the cell lysate and measured by a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). The final concentration of DMSO was 0.2% and 0.3% for the agonistic and co-exposure tests, respectively. All tests were performed in at least three independent experiments, each in triplicate. The cytotoxicity was examined by visually inspecting the cells under the microscope before cell lysis.

Table S1 shows the characteristics of the seven reporter gene cell lines. In general, the cell lines performed similarly as in the previous studies *i.e.* similar EC_{50} and fold induction[14,19,22], except a reduction of the maximal fold induction seen for TGRM (from around 2000 fold to 320 fold), and an increase of EC_{50} of PR-TM ($705 \pm 40 \times 10^{-9}$ M compared to 1.5×10^{-9} M), however this latter value was close to that observed in a recent study of $3.3 \pm 0.9 \times 10^{-7}$ M[23].

Table S1. Characteristics of the seven reporter gene cell lines and the co-exposure EC_{50} concentrations.

Names	Original cell type	Receptor	Reference compound	EC_{50} (M) (0.2% DMSO)	EC_{50} (M) (0.3% DMSO)	FI_{max}	Co-exposing EC_{50} (M)
DR-H4IIE	H4IIE: rat hepatoma	AhR	TCDD	$25 \pm 3 \times 10^{-12}$	$12.5 \pm 2 \times 10^{-12}$	13	15×10^{-12}
DR-HepG2	HepG2: human hepatoma	AhR	TCDD	$624 \pm 21 \times 10^{-12}$	$658 \pm 28 \times 10^{-12}$	12	650×10^{-12}
DR-T47-D	T47-D: human carcinoma mammary gland	AhR	TCDD	$111 \pm 14 \times 10^{-12}$	$100 \pm 38 \times 10^{-12}$	9	150×10^{-12}
ER-MMV	MCF-7: human breast cancer cell	ER	17β-estradiol (E2)	$5.3 \pm 1 \times 10^{-12}$	$3.2 \pm 2 \times 10^{-12}$	4	12.5×10^{-12}
TGRM	T47-D: human carcinoma mammary gland	GR	dexamethasone (Dex)	$6.2 \pm 1 \times 10^{-9}$	$4.5 \pm 1 \times 10^{-9}$	320	10×10^{-9}
PR-TM	T47-D: human carcinoma mammary gland	PR	progesterone (P4)	$705 \pm 37 \times 10^{-9}$	$665 \pm 46 \times 10^{-9}$	183	1000×10^{-9}
TARM	T47-D: human carcinoma mammary gland	AR	5α-dihydrotestosterone (DHT)	$520 \pm 180 \times 10^{-12}$	$176 \pm 36 \times 10^{-12}$	16	400×10^{-12}

EC_{50}: Concentration causing half-maximal response of the reference compound. FI_{max}: maximum fold induction of the response of the reference compounds compared to the response of the vehicle DMSO. Co-exposing EC_{50}: co-exposing concentration of the reference compound in the antagonistic tests.

2.5. EROD assays in DR-HepG2 and DR-T47-D

The EROD assay measures ethoxyresorufin-O-deethylase (eroD) enzyme activity that is associated with CYP1A converting ethoxyresorufin to fluorescent resorufin. They were used to determine the CYP1A activity of the mixtures in DR-HepG2 and DR-T47-D. The EROD assays were performed according to Schiwy *et al*., (2015)[24] with the exposure conditions as described in CALUX assays. However, to adjust to the much higher TCDD sensitivity of the EROD assays, the maximum TCDD concentrations were 20 and 40-fold lower (500 pM for DR-HepG2 and 250 pM for DR-T47-D) than in CALUX assays (10 nM for both), and the corresponding TCDD EC_{50} co-exposure in the antagonistic tests was respectively 100 pM and 50 pM, instead of 650 pM and 150 pM in CALUX assays. After 24h exposure, 100 µl of working solution, freshly prepared by dissolving 7-ethoxyresorufin (5 µM) (Sigma Aldrich) in DMEM without phenol red, was added into 50 µl lysed-cell extract, followed by an incubation for 30 minutes at 37°C in the dark. To terminate the reaction, 100 µl chilled to 4$_o$C methanol (Merck, Massachusetts, USA) was added. The fluorescence signal of resorufin was measured at 550/595 nm using a Perkin Elmer VICTOR X5 MultiLabel Plate Reader, Germany.

2.6. Calculations of relative responses RR, EC_{50}, IC_{50}, and efficacy

The cells' responses were obtained as relative light units (RLUs). The activities of the compound/mixture (n) for both CALUX and EROD assays were presented as relative responses ($RR_{a(n)}$ and $RR_{i(n)}$ (%) for agonistic and co-exposure tests, respectively and determined as:

$$(1)\ RR_{a(n)} = \frac{R_{a(n)}}{R_{ref-max}} \times 100$$

$$(2)\ RR_{i(n)} = \frac{R_{i(n)}}{R_{ref-EC50}} \times 100$$

$RR_{a(n)}$ (Eq. 1) or $RR_{i(n)}$ (Eq. 2) was the percentage (%) of the agonistic ($R_{a(n)}$) or co-exposure ($R_{i(n)}$) response of the compound/mixture (n) compared to the maximum response ($R_{ref-max}$) or to the response of the spike-in EC_{50} ($R_{ref-EC50}$) of the reference. A value of $RR_{i(n)}$ (%) higher than 100% reflects an additive/enhancing action of the compound/mixture on top of the effect caused by the presence of the reference compound at EC_{50}. Meanwhile, a value less than 100% quantitates the antagonistic potential of the compound/mixture to inhibit the activity of the reference compound at EC_{50}, thus, the lower the $RR_{i(n)}$ (%) is, the stronger the antagonistic activity.

The dose-response curves were generated by fitting a four-parameter non-linear stimulation or inhibition regression for agonistic (Eq. 3) or antagonistic (Eq. 4) tests:

$$(3)\ RR_{a(n)x} = B + \frac{x^H (T - B)}{x^H + {EC_{50}}^H}$$

$$(4)\ RR_{i(n)x} = B + \frac{T - B}{1 + \frac{x^H}{{IC_{50}}^H}}$$

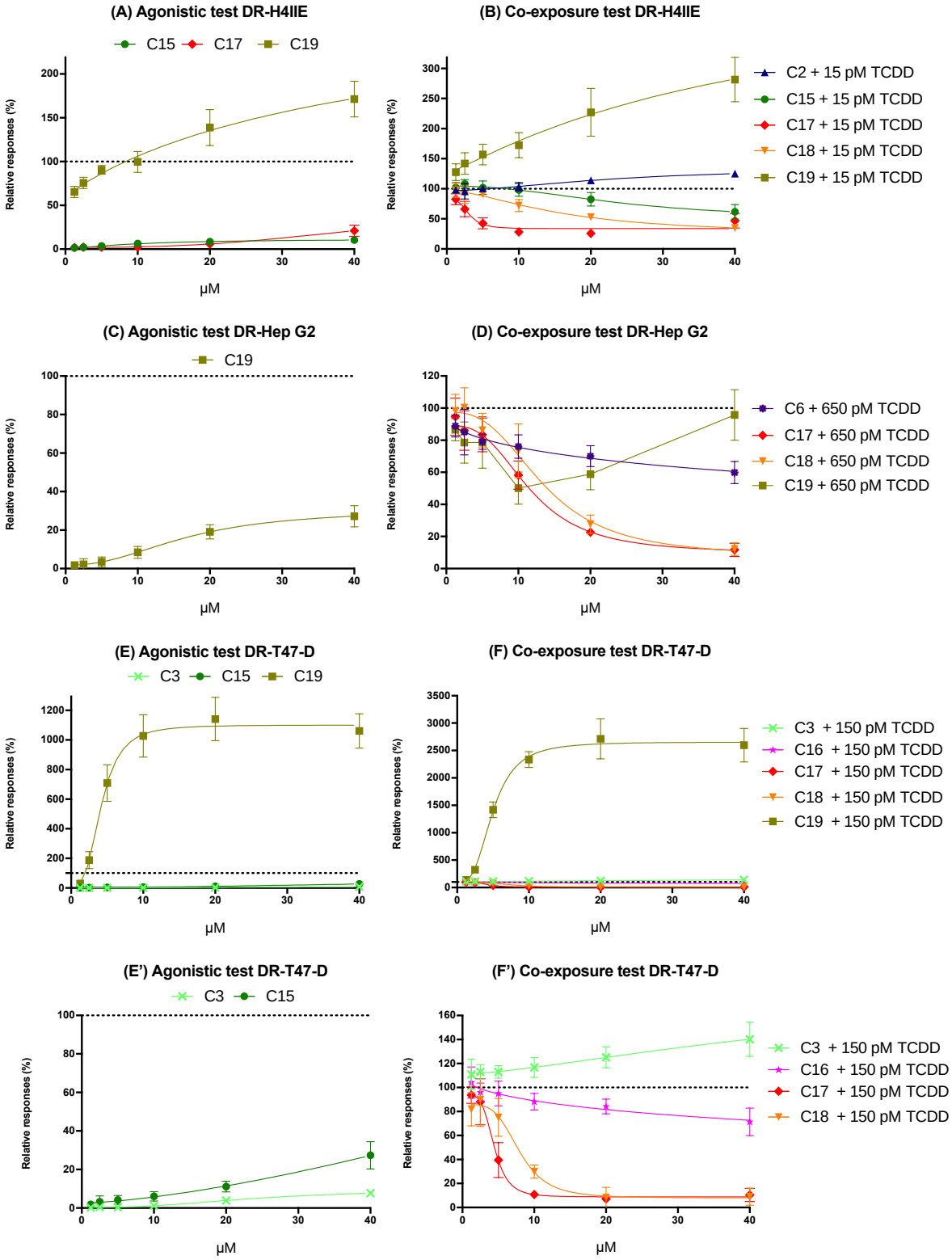
where *x* is the concentration of a tested compound/mixture inducing the relative response $RR_{a(n)x}$ or $RR_{i(n)x}$. EC_{50} and IC_{50} are the half maximal effective concentrations for an agonist and antagonist, respectively[25]. B = bottom, T = Top, H = Hillslope. B was constrained higher than 0, respectively for the minimum and maximum responses in agonistic and antagonistic tests. A compound/mixture was considered active if its effect was significantly different from that of DMSO alone (ANOVA, Graphpad PRISM, $p < 0.05$). The efficacy ($RR_{a/max}$ or $RR_{i/max}$ (%) for agonistic or antagonistic activities, respectively) was determined as the significant maximum effect of the compound/mixture. When no full dose response curve was obtained, $RR_{a/max}$ or $RR_{i/max}$ was assigned as the effect induced by the highest tested concentration of the compound/mixture.

3. Results

3.1. CALUX activities of the 18 compounds

No cytotoxicity was recorded for any of the 18 compounds within the concentration range tested for both agonistic and co-exposure experiments. Among the pesticides, the most abundant compound C1 (desphenyl-chloridazon) did not show any activity, while C2 (methyl-desphenyl-chloridazon), C3 (desethylatrazine), and C6 (atrazine) displayed activity in at least one cell line (Tables 2 and 3). C2 and C3 exerted a weak agonistic response that was best observed when cells were cotreated with EC_{50} TCDD, respectively in DR-H4IIE and DR-T47-D (Figures 1B and 1F'), while C6, the parent compound of C3, antagonized the EC_{50} TCDD activity in DR-HepG2 with $RR_{i/max}$ = 70% (Figure 1D). The most active pesticide was C15 (chlorpyrifos), inducing both weak agonistic ($RR_{a/max}$ = ~10%) and significant antagonistic responses in DR-H4IIE (Figures 1A and 1B) and ER-MMV (Figures 1G and 1H) ($RR_{i/max}$ = 62% and 43%, respectively), while it was only agonistic ($RR_{a/max}$ = 27%) in DR-T47-D (Figure 1E'), and only antagonistic ($RR_{i/max}$ = 68%) in PR-TM (Figure 1J).

C16 (BPA) strongly induced the response of ER-MMV with a $RR_{a/max}$ = 119%, higher than the reference compound E2 (Figure 1G), but did not cause any effect when cells were co-exposed with EC_{50} E2. It also inhibited the responses of the four T47D-derived transgenic lines in the co-exposure tests, moderately in DR-T47D and TGRM (Figures 1F' and 1I), and particularly strongly in PR-TM and TARM with $RR_{i/max}$ = 15% (Figures 1J and 1K).


(A) Agonistic test DR-H4IIE
C15
C17
C19
Relative responses (%)
μM
(B) Co-exposure test DR-H4IIE
C2 + 15 pM TCDD
C15 + 15 pM TCDD
C17 + 15 pM TCDD
C18 + 15 pM TCDD
C19 + 15 pM TCDD
(C) Agonistic test DR-Hep G2
C19
(D) Co-exposure test DR-Hep G2
C6 + 650 pM TCDD
C17 + 650 pM TCDD
C18 + 650 pM TCDD
C19 + 650 pM TCDD
(E) Agonistic test DR-T47-D
C3
C15
C19
(F) Co-exposure test DR-T47-D
C3 + 150 pM TCDD
C16 + 150 pM TCDD
C17 + 150 pM TCDD
C18 + 150 pM TCDD
C19 + 150 pM TCDD
(E') Agonistic test DR-T47-D
(F') Co-exposure test DR-T47-D

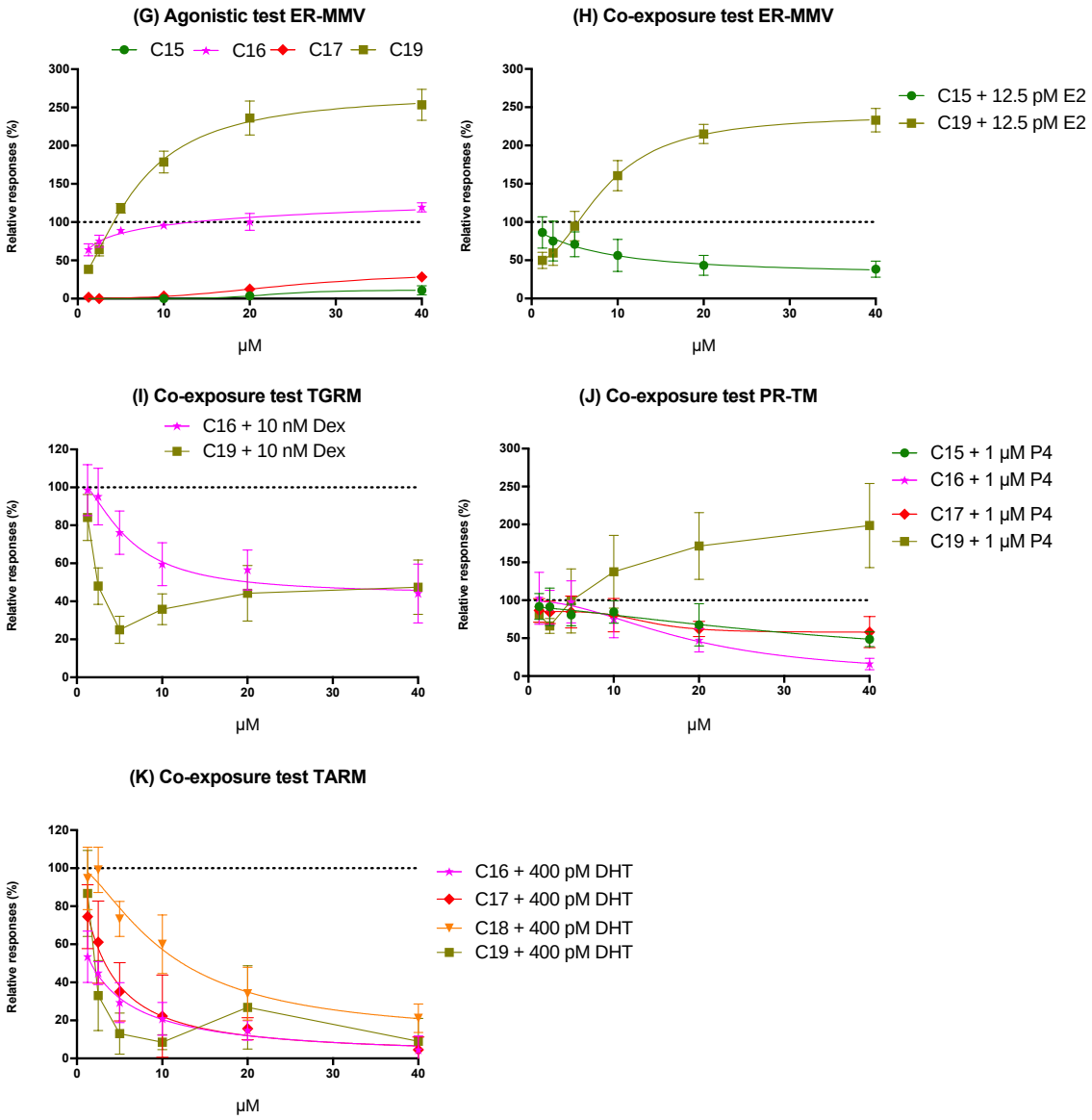


Figure 1. Dose-response curves of the active chemicals in (A-B) DR-H4IIE, (C-D) DR-HepG2, (E-F') DR-T47-D, (G-H) ER-MMV, (I) TGRM, (J) PR-TM, and (K) TARM, inducible respectively by aryl hydrocarbon, estrogen, glucocorticoid, progesterone, and androgen receptors. (Mean ± SD, n = 3, 0.2% or 0.3% DMSO for agonistic or co-exposure tests, respectively). Dash lines (100%) are the maximum response or the response of the co-exposing EC_{50} of the corresponding reference compound in agonistic or co-exposure tests, respectively.

The most potent group was PAHs, which caused a response in all seven cell lines, except C18 which displayed no response in ER-MMV, TGRM, and PR-TM. Interestingly, C17 (fluoranthene) and C18 (phenanthrene) behaved differently from C19 (BaP). While C18 did not induce agonistic activities in any cell line, C17 caused a weak agonistic response, with $RR_{a/max}$ = 21% and 28% in DR-H4IIE and ER-MMV, respectively (Table 2). However, they both strongly inhibited the EC_{50} TCDD response with $RR_{i/max}$ = ~40% in DR-H4IIE, and even more

strongly in DR-HepG2 and DR-T47-D ($RR_{i/max}$ = ~10%). C17 also antagonized P4 in PR-TM and DHT in TARM with $RR_{i/max}$ = 62% and 16%, respectively, whereas C18 inhibited only the TARM response with $RR_{i/max}$ = 21% (Table 3).

C19 (BaP) was a potent agonist in DR-H4IIE with $RR_{a/max}$ = 171%, nearly twice the cells' maximum response to TCDD (Figure 1A). The effect remained when it was co-exposed with EC_{50} TCDD with $RR_{i/max}$ = 227% (Figure 1B), since this resulted in a final $RR_{a(C19)/max}$ = 126% after the spike-in EC_{50} reference was subtracted and the response $RR_{a(C19)}$ was compared to the maximum response of the reference compound. C19 was also agonistic to AhR in human hepatoma DR-HepG2 cells, but to a lesser extend ($RR_{a/max}$ = 27%) (Figure 1C), causing a partially antagonistic activity in this cell line when co-exposed with EC_{50} TCDD, showing an antagonistic activity up to 10 μM and an agonistic activity at higher concentrations (Figure 1D). Surprisingly, C19 induced an extremely strong agonistic response in human mammary gland carcinoma DR-T47-D, with $RR_{a/max}$ = 1061% (Figure 1E), which remained similar in the co-exposure test with $RR_{i/max}$ = 2713% or a final $RR_{a(C19)/max}$ = 1266% (Figure 1F), indicating that the presence of TCDD did not affect the C19 activity in this cell line.

In ER-MMV, C19 induced a strong agonistic response of $RR_{a/max}$ = 253%, while in the presence of EC_{50} E2, an inhibition was observed at lower concentrations and an activation at higher concentration, parallel to the agonistic curve with a $RR_{i/max}$ = 233% or $RR_{a(C19)/max}$ = 102%, less than half of $RR_{a/max}$ (Figure 1G and 1H). It also caused a similar response in PR-TM, but only when co-exposing the cells with P4, with a $RR_{i/max}$ = 172% or $RR_{a(C19)/max}$ = 111% (Figure 1J). Moreover, C19 significantly antagonized both Dex and DHT in TGRM and TARM cells, with $RR_{i/max}$ = 44% and 9%, respectively. All data are summarized in tables 2 and 3.

3.2. CALUX activities of the mixtures

No activity was observed for the PFC mixture. In DR-H4IIE, the ED mixture showed an agonistic activity similar to that of the PAH mixture with $RR_{a/max}$ = 42% and 45%, respectively, while the PEST mixture activation was lower ($RR_{a/max}$ = 17%). In comparison, the ER-MMV responded similarly to both the PAH and PEST mixtures with $RR_{a/max}$ = 17% and 16%, respectively, while the ED mixture caused a strong agonistic effect ($RR_{a/max}$ = 32%) (Table 2).

Additive responses of these mixtures were observed when the cells were co-exposed with TCDD or E2 (Table 3). However, the additive responses were not significant in DR-H4IIE with only $RR_{i/max}$ = ~125% for both ED and PAH mixtures, and only 115% for the PEST mixture. A similar result was obtained in ER-MMV ($RR_{i/max}$ PAH and ED = 145% and PEST = 122%).

Table 2. EC_{50} and $RR_{a/max}$ values of the agonistic responses to the selected 18 chemicals and four mixtures (n = 3, 0.2% DMSO).

Name	Compound	DR-H4IIE		DR-HepG2		DR-T47 D		ER-MMV	
		EC_{50} (μM) ± SE	$RR_{a/max}$ (%)	EC_{50} (μM) ± SE	$RR_{a/max}$ (%)	EC_{50} (μM) ± SE	$RR_{a/max}$ (%)	EC_{50} (μM) ± SE	$RR_{a/max}$ (%)
C1	DPC	-	-	-	-	-	-	-	-
C2	Me-DPC	-	-	-	-	-	-	-	-
C3	Desethylatrazine	-	-	-		24.1 ± 4.8	8	-	-
C5	Metolachlor ESA	-	-	-	-	-	-	-	-
C6	Atrazine	-	-	-	-	-	-	-	-
C7	BH479-8	-	-	-	-	-	-	-	-
C8	1H-benzotriazole	-	-	-	-	-	-	-	-
C9	PFOS	-	-	-	-	-	-	ND	7
C10	PFOA	-	-	-	-	-	-	-	-
C11	PFHxA	-	-	-	-	-	-	-	-
C12	PFHpA	-	-	-	-	-	-	-	-
C13	PFHxS	-	-	-	-	-	-	ND	6
C14	Simazine	-	-	-	-	-	-	-	-
C15	Chlorpyrifos	10.5 ± 2.3	10	-	-	ND	27	22.6 ± 17.7	11
C16	BPA			-	-	-	-	1.2 ± 0.1	119
C17	Fluoranthene	46.5 ± 94	21	-	-	-	-	25.4 ± 6.4	28
C18	Phenanthrene	-	-	-	-	-	-	-	-
C19	BaP	44 ± 59	171	16.8 ± 2.3	27	4.2 ± 0.2	1061*	6.5 ± 0.4	253*
PFAA		-	-	-	-	-	-	-	-
PAH		ND	45	-	-	-	7	ND	17
PEST		ND	17	-	-	-	-	ND	16
ED		ND	42	ND	6	ND	11	ND	32

EC_{50}: the concentration inducing half of the maximum activation response; SE: Standard Error; $RR_{a/max}$: observed activation efficacy; ND: Not Determined. -: no response. *Maximum efficacy observed within the tested concentrations.

Table 3. IC_{50} and $RR_{i/max}$ values of the co-exposure responses to the selected 18 chemicals and four mixtures (n = 3, 0.3% DMSO).

Name	Compound	DR-H4IIE		DR-Hep G2		DR-T47 D		ER-MMV		TGRM		PR-TM		TARM	
		IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$	IC_{50} (µM)	$RR_{i/max}$
C1	DPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2	Me-DPC	23 ± 14	125*	-	-	-	-	-	-	-	-	-	-	-	-
C3	Desethylatrazine	-	-	-	-	ND	140	-	-	-	-	-	-	-	-
C5	Metolachlor ESA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C6	Atrazine	-	-	ND	70*	-	-	-	-	-	-	-	-	-	-
C7	BH479-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C8	1H-benzotriazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C9	PFOS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C10	PFOA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C11	PFHxA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C12	PFHpA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C13	PFHxS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C14	Simazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C15	Chlorpyrifos	24 ± 10	62	-	-	-	-	6.8 ± 5.2	43*	-	-	44 ±	68*	-	-
C16	BPA	-		-	-	ND	71	-	-	5.8 ± 1.7	44	18.5 ±	15	4.3 ± 3	15*
C17	Fluoranthene	3 ± 0.3	47	11.6 ±	12	4.4 ± 0.3	8	-	-	-	-	13.9 ±	62*	3.2 ± 2.6	16*
C18	Phenanthrene	15 ± 4	35	12.3 ±	12	7.9 ± 0.6	7	-	-	-	-	-	-	10.4 ±	21
C19	BaP	43.2 ±	227*	11.5 ±	50*	4.9 ± 0.2*	2713*	8.4 ± 0.5*	233	2.3 ± 881	44*	10.5 ± 3*	172*	2.3 ± 29	9*
PFAA		-	-	-	-	-	-	-	-	-	-	-	-	-	-
PAH		512 ± 241	124	-	-	-	-	602 ±	145	-	-	-	-	-	-
PEST		ND	115	-	-	-	-	ND	122	-	-	-	-	-	-
ED		571 ± 200	126	-	-	-	-	ND	145	-	-	-	-	-	-

IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; $RR_{i/max}$: observed inhibition efficacy; ND: Not Determined. -: no response. *Maximum efficacy observed within the tested concentrations.

Since full dose response curves were not obtained within the tested concentration range, no values could be deduced for EC_{50}, while some IC_{50} values have been determined (Tables 2 and 3).

From table 2, it seems that the observed agonistic effects of the PAH and the ED mixtures in DR-H4IIE were due to the agonistic activity of C19, while the agonistic effects seen in ER-MMV were due to the combination of C16 and C19. Therefore, a test was performed using the concentrations of C19, C16 or C16+C19 corresponding to their concentrations in the mixtures, to confirm this assumption. The agonistic activity of C19 explained most of the agonistic activity of PAH and ED mixtures in DR-H4IIE as shown in Figure 2A1, while the combined agonistic effects of C16+C19 explained most of the agonistic effect of the ED mixture in ER-MMV cells (Figure 2A2). No major contributor could be identified for the weak agonism of the PEST mixture in DR-H4IIE.

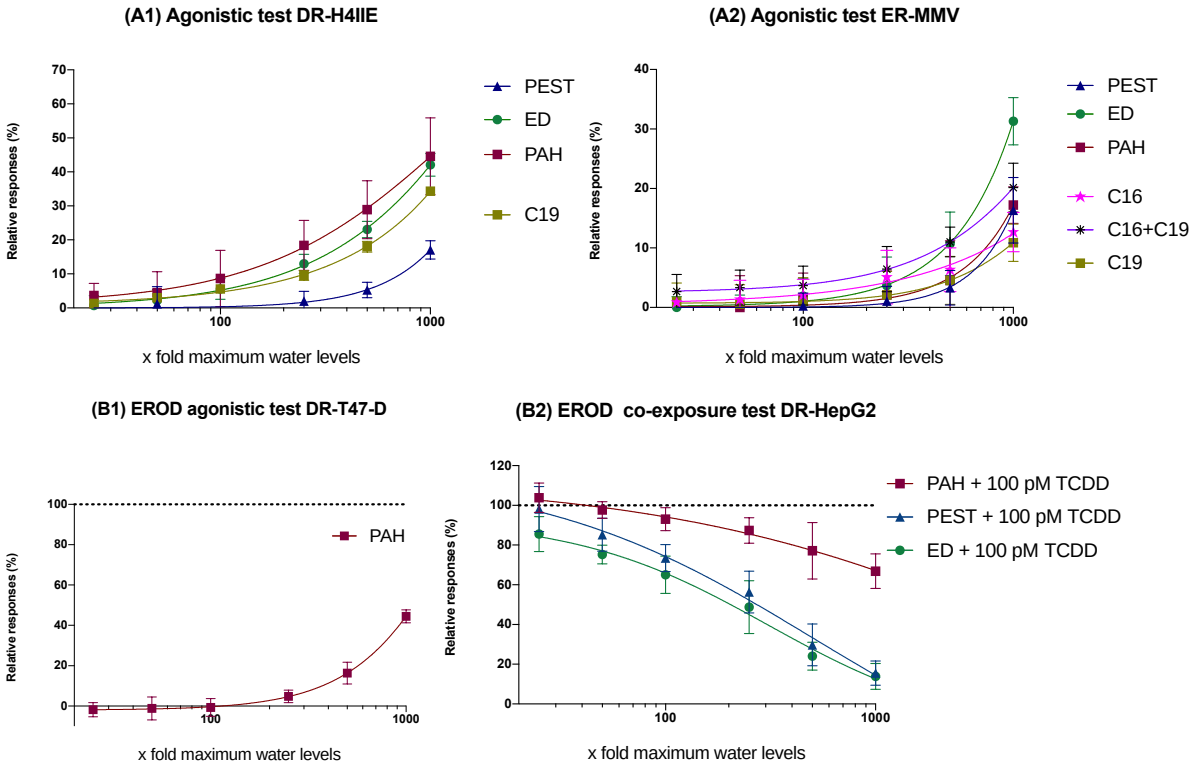


Figure 2. (A) Dose-response curves of the mixtures and C16, C19 and C16+C19 in the DR-H4IIE (A1) and ER-MMV (A2). (B) Dose-response curves of the mixtures in DR-T47-D (B1) and DR-HepG2 (B2) in EROD assays. (Mean ± SD, n = 3, 0.2% or 0.3% DMSO for agonistic or co-exposure tests, respectively). Dash lines (100%) are the maximum response or the response of the co-exposing EC_{50} of the corresponding reference compound in agonistic or co-exposure tests, respectively.

3.3. EROD assays with the mixtures in DR-HepG2 and DR-T47-D

Recently, it was shown that EROD assays could be used as a sensitive alternative to reporter gene assays for AhR activity[26]. Thus, we decided to confirm the activity of the mixtures on the two human cell lines (DR-HepG2 and DR-T47-D) using this approach. In DR-T47-D, while only an agonistic effect was observed for the ED mixture in CALUX assays, there was a moderate agonistic response seen for the PAH mixture with $RR_{a/max}$ = 45% (Figure 2B1) and weak antagonistic responses seen for the PEST and ED mixtures ($RR_{i/max}$ < 80%) (data not shown). The PEST and ED mixtures induced the same antagonistic responses in the DR-HepG2 with IC_{50} PEST = 180 ± 25 and IC_{50} ED = 177 ± 23 fold and $RR_{i/max}$ PEST = 16% and $RR_{i/max}$ ED = 14%, while the PAH mixture displayed a weaker response of $RR_{i/max}$ = 67% (Figure 2B2).

4. Discussion

4.1. Endocrine disrupting activities of the 18 compounds

This study is one of the few studies[22,23,27,28] using a panel of multiple cell-based bioassays (seven reporter gene cell lines including five receptors (AhR, ER, GR, PR, and AR)) for *in vitro* profiling of the potential endocrine disrupting activity of multiple chemicals. Eighteen compounds frequently detected in Wallonia raw water intended for drinking water production were tested, belonging to several groups (pesticides, 1H-benzotriazole, PFCs, BPA, and PAHs). Appearing with relatively high frequency of quantification (FOQ) in water, several herbicides (C1, C5, C7, C14) and C8 (FOQ >~40%), and PFCs (C9-C13) (FOQ = ~20%) did not display a significant response in these bioassays, except a weak response ($RR_{a/max}$ < 10%) for C9 and C13 in ER-MMV. Hence, they are unlikely to induce any endocrine disrupting activity via these receptors. Three compounds (C2, C3 and C6) triggered an AhR response in a species- and cell type-specific manner. To our best knowledge, these results are the first recorded for these cell lines. The compounds were frequently detected in raw water samples; hence their activities may be a matter of concern. Meanwhile, C15, C16, C17, C18 and C19 were active on several receptors. AhR and ER were more general targets for both agonistic and antagonistic activities than the other receptors, responding to respectively eight and six compounds, while GR, PR and AR were targets only for antagonism (see Figure S1 for detailed clusters).

C15 (chlorpyrifos) caused both agonistic and antagonistic activities towards AhR and ER, and only antagonized PR. Therefore, C15 appears to be a potential ED. The result was consistent with previous *in vitro* reports concerning this compound showing activation of AhR[29] and ER[30–32] and with *in vivo* estrogenic activity observed in mammary gland in female rats[33]. The EU

legislation imposes a limit of 0.1 μg/L in drinking water for total pesticides[7], which is equivalent to 0.3 pM chlorpyrifos, 10,000 fold lower than the lowest observed effect concentration of chlorpyrifos at 5 μM (Figures 1B, E', H, and J). However, due to a high bioconcentration factor (*e.g.* 100 to 5100 in fish[34]), a high documented toxicological activity[32,33,35], and a considerably high contamination potential due to the current use in the US and a very recent ban in the EU, the monitoring of chlorpyrifos should be specifically indicated in the legislation.

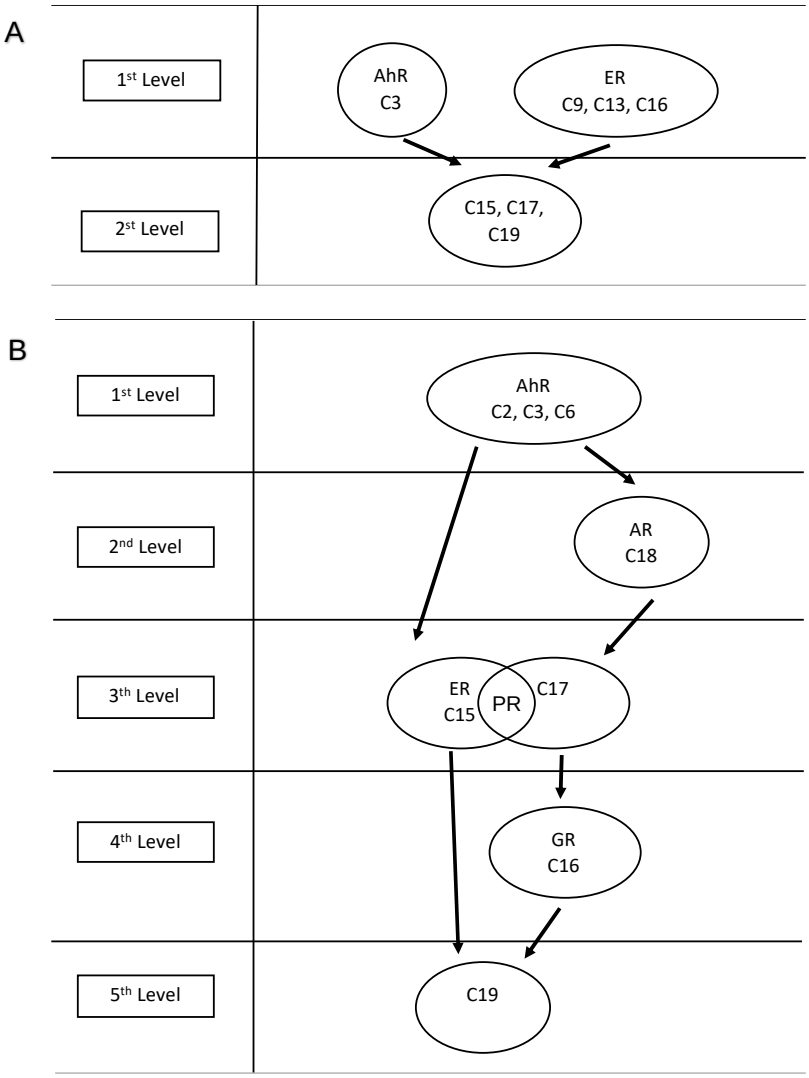


Figure S1. Clustering of compounds inducing an (A) agonistic activity and (B) antagonistic/additive activity on one or several receptors. AhR: rat and/or human aryl hydrocarbon receptor; ER: human estrogen receptor; PR: human progesterone receptor; GR: human glucocorticoid receptor; AR: human androgen receptor. The compounds which showed either agonistic or antagonistic/additive effects towards any of the five receptors, were grouped into clusters of, respectively two and five levels corresponding to the number of receptors they act on. In level 1, each circle displays the name of the compound and the receptor induced by the compound. In level n+1, the circle gives the same information as in

the previous level, and, if the receptor was mentioned in the previous level, connecting arrows are used to indicate the relationship between the compounds and the receptors.

BPA (C16) is an ubiquitous contaminant, prevalent also in water[36–38], whose ability to activate ER-mediated pathways is well documented[39–41] and has been confirmed in this study. However, its activity does not always involve estrogen-mediated mechanisms as shown in human endometrial stromal fiboblasts[42] or in human endometrial adenocarcinoma cells where it prevented the proteasome-mediated degradation of ERβ[43]. In addition, BPA inhibited the activities of all the reference compounds in the T47-D transgenic cell lines: DR-T47-D, TGRM, TARM, and PR-TM, while only anti-androgenic was previously shown in *in vitro* reporter gene assays[27,28,44]. BPA was present in Wallonia raw water at C_{max} = 31 ng/L but rarely detected (FOQ = 9.1%)[8]. Currently, BPA does not appear to pose a threat to these drinking water sources. However, due to its significant activity, BPA should be listed for monitoring in the Council Directive 98/83/EC[7].

PAHs are a diverse class of toxicants. They activate AhR[45–47] as one of their major toxicity pathways and interfere with estrogenic signaling through direct or indirect estrogen-like effects[48,49] and anti-estrogenic effects[49,50]. We not only confirmed the finding of weak, but significant induction of the rat AhR response to C17 (fluoranthene) (but not for C18 (phenanthrene) in DR-H4IIE[47], but also showed that C17 and C18 strongly antagonized TCDD-induction in the human DR-HepG2 and DR-T47-D. We also newly discovered the strong antagonistic effects of both C17 and C18 towards androgens in TARM (Table 3). Neither fluoranthene nor phenanthrene are in the list of the EU legislation for water intended for human consumption[7]. Regarding their significant endocrine disrupting activities, especially anti-AhR and anti-AR towards human-based cell lines and their presence in water sources, we propose to add fluoranthene and phenanthrene to the list of raw water monitoring in the EU.

C19 (BaP) is known as a potent endocrine and growth disruptor, particularly altering the AhR pathway and displaying both estrogenic and anti-estrogenic activity in several systems[51,52]. In this study, BaP significantly modified the transcriptional activities of all five receptors in all seven cell lines. It was a strong AhR agonist in DR-H4IIE and caused both agonistic and partially antagonistic activities in DR-HepG2, as documented previously[45,46]. BaP also displayed an agonistic and a partial antagonistic activity in ER-MMV. It was shown that the estrogenic activity of BaP in MCF-7 cells required the activation of AhR to produce estrogenic BaP metabolites, most notably 3-OH-BaP[53]. Indeed, BaP failed to stimulate ER-dependent

reporter luciferase activity in AhR-knockout MCF-7 cells, indicating that the estrogenic-activity of BaP is a “secondary” response driven by its activation of the AhR signaling pathway.

However, the most striking result was the enhanced response observed in DR-T47D exposed to BaP with a response 10-fold higher than the maximum response to TCDD. A “pure” AhR antagonist, GNF315[54] at 10 μM inhibited the BaP-induced AhR activation in DR-T47-D by more than half, from $RR_{a/max}$ = 1061% to $RR_{a/max}$ = 346% (Figure S2). However, the other AhR antagonist, CH-223191 did not affect the BaP activity. CH-223191 had also failed to inhibit the stimulation of AhR-DNA binding of beta-naphthoflavon (a PAH like chemical)[55]. Meanwhile, ER antagonists (fadrozole and fulvestrant) at 1 μM did not affect the cells' response to BaP (data not shown). Thus, the BaP-induced enhancement of AhR activation seems to be partially dependent on the activity of the AhR, but unrelated to the activity of ER. This enhancement could be due to some mechanism acting downstream of AhR activation that is triggered by BaP specifically in mammary gland cells. This is consistent with the observed enhancement (2.5-fold) of ER activation in ER-MMV, also derived from human mammary gland cells (MCF-7). T47-D cells are also used in the reporter cell-assays for progesterones, glucocorticoids and androgens. We also observed an enhanced response (about 2-fold) to progesterones in PR-TM, consistent with a specific enhancement mechanism in these cells, but antagonism against glucocorticoids and androgens. It is important to mention here that the parent T47-D cells produce their endogenous PR, which is thus also present in the TGRM and TARM that contain a transgene coding, respectively for exogenous GR or AR. This explains why the initial antagonism of GR and AR observed in these lines at lower BaP concentrations is dominated at higher concentrations by the enhanced PR response. Further investigations will be required to fully understand the actions of BaP in these cells.

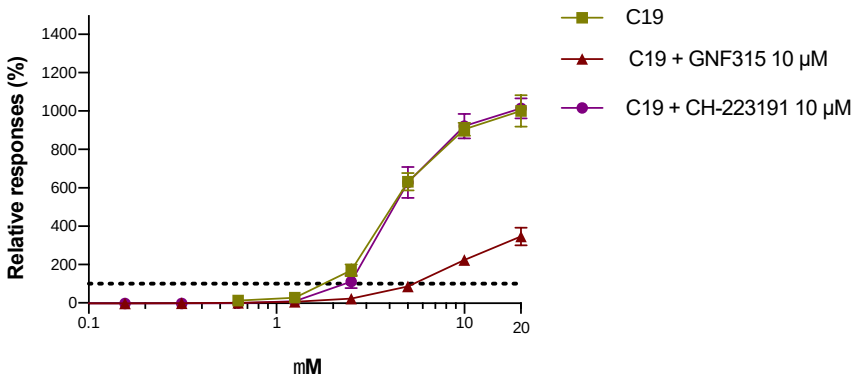


Figure S2. Dose response curves of DR-T47-D cells co-exposed to C19 and AhR antagonists (GNF315 or CH-223191) after 24h exposure (Mean ± SD, n = 3, 0.3% DMSO). Dash line (100%) is the TCDD maximum response.

Such an enhancement is extremely concerning in terms of human exposure to BaP which is mostly via food consumption rather than via drinking water. BaP exposure results in a maximal response (10-fold higher than TCDD) at only 10 μM, while inductions similar to that of TCDD are already observed at 2.5 μM in the human mammary gland DR-T47-D cells. Although BaP is rapidly metabolized and thereby quickly eliminated from the body[56], BaP is still the ubiquitous PAH contaminants in human samples[57]. BaP contamination can be found at 7.1 nM in the follicular fluid of women who smoke[58] or 20 nM in serum samples from autopsy cases of African American females[57]. Therefore, a 100-fold increased exposure up to 2.5 μM of BaP in blood could be realistic due to the chronic exposure of highly exposed sub-populations or acute exposure due to food contamination incidents.

4.2. Activities of the mixtures

The ED, PAH, and PEST mixtures caused an agonistic effect on AhR in DR-H4IIE and on ER in ER-MMV. The AhR agonistic effect of ED and PAH could be reproduced using only their main component, C19, while the very weak activity of the PEST mixtures was probably due to the addition of the activities of its components, each individually being too weak to be detected. In contrast, the ER agonistic activity can be attributed to the presence of either C16 (in PEST), C19 (in PAH), or both (in ED).

None of the mixtures displayed any effect in either DR-HepG2 or DR-T47-D in CALUX assays, except for a weak agonistic effect of the ED mixture for both cell lines (Table 3 and 4). In contrast, EROD assays revealed a significant antagonistic effect for the three mixtures (PAH, PEST and ED) in DR-HepG2 and a significant agonistic effect for the PAH mixture in DR-T47-D (Figure 2B). The micro-EROD assays developed by Schiwy *et al*., (2015)[24] revealed the highest sensitivity to TCDD, allowing the detection of lower effective concentrations compared to CALUX assays[26]. This may be due to the fact that 7-ethoxyresorufin O-dealkylation can be catalyzed in humans by two enzymes, CYP1A1 and CYP1A2[59], encoded by endogenous AhR target genes, while CALUX assays measure AhR-induced expression of an artificially inserted reporter gene. Moreover, the co-exposing TCDD EC_{50} for EROD was several times lower than that for CALUX assays, which makes the detection of antagonism of the mixtures easier. This greater sensitivity of the EROD assays for measuring CYP1A activity in the two human cell lines allows for better detection of agonistic activities of the mixtures, but also leads to better sensitivity for antagonistic activities, due to the lower concentration of the co-exposed TCDD at their respective EC_{50}. Note that, contrary to CALUX assays, however, antagonism in EROD assays can also result from inhibition of the enzyme activity, which is impossible to detect in the reporter assay.

Conclusions

We tested the endocrine disrupting activities of 18 compounds shown to be present at trace concentrations in raw water intended for drinking water production in Wallonia. Both pure solutions of each compound and mixtures were studied for their ability to agonize and antagonize the transcriptional activities of a panel of five receptors, listed as AhR, ER, AR, PR and GR. Among these compounds, chlorpyrifos, BAP, fluoranthene, phenanthrene, and BaP demonstrated significant activities which involved more than one mechanism. Their presence in raw water source should be monitored more strictly. BAP as well as fluoranthene and phenanthrene should be added to the list of monitoring due to their significant endocrine disrupting activities. BaP alone or with BPA were the main contributors for the agonistic activities of the ED and PAH mixtures towards the AhR in DR-H4IIE and ER in ER-MMW. EROD assays were able to reveal the antagonistic effect of the mixtures in DR-HepG2 cells, which has not been observed in CALUX assays. The results revealed the multi-endocrine disrupting activities of several contaminants in raw water intended for drinking water production, some of them not being mentioned in the EU legislation relating to water monitoring. Their (anta-)agonistic effects on different receptors with the potential for additive or inhibitory effects in the mixtures should be considered in their risk assessment.

Conflict of interest: The authors declare that there is no conflict of interest.

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Chapter 5: Discussion, General Conclusions and Future Direction

1. The AhR antagonistic activity of the persistent organic pollutants (POPs) prevalent in Scandinavian human blood

Our study showed that, among the 29 compounds included in the total POP mixture derived from blood levels found in a Scandinavian population, 16 chemicals exerted antagonistic effects on AhR, while only four showed AhR agonism. Their antagonistic activities were AhR-dependent and seemed to act through competition with TCDD for the same binding site, except for *t*-nonachlor and α-chlordane. Such AhR antagonistic activities of POPs had been previously observed in several screening studies[1,2] and mixture studies[3,4]. As expected, the total POP mixture, and sub-mixtures thereof were found to induce only antagonistic effects on AhR. Moreover, our antagonistic tests indicated that the total POP mixture would antagonize the activity of the dioxin like-compounds contaminating the Scandinavian population in real life.

Since its discovery, the AhR has been considered as a master receptor in xenobiotic metabolism and toxicity due to the fact that it mediates the effects of the halogenated aromatic hydrocarbon environmental pollutants. Therefore, the AhR agonistic activity of POPs especially dioxins, has been studied for decades, while the antagonistic counterpart has not yet received much attention, especially regarding its physiological consequences on an organism's health.

However, increasing evidence supports important roles of AhR in normal development and homeostasis, while toxicity induced by AhR xenobiotic ligands could be due to perturbation of these normal processes[5]. Studies in cell lines and rodent models pointed out that the deregulation of these AhR dependent pathways is associated with several life-threating diseases such as cancer, immune and cardiovascular disorders, and infertility[6,7]. For example, AhR knockout (KO) mice display various developmental abnormalities, highlighting the roles of AhR in perinatal growth[8,9], regulation of blood pressure, central nervous system, cell proliferation, production of peripheral lymphocyte counts[10,11], and in female fertility[9].

On the other hand, AhR is also induced by a wide range of endogenous and natural ligands, which may actually contribute to normal physiological functions. Organisms are exposed to a complex mixture of compounds via their diet. The diet provides nutrients and/or other

physiological values, but some components may be toxic for the animals. A biological 'cocktail' of endogenous, pseudo-endogenous (pseudo-endogenous AhR ligands are defined as any metabolite that is produced by the gut microbiota and capable of binding to the AhR[12]) and dietary AhR ligands exists, suggesting their predominance in modulating AhR biological functions. Dietary derived AhR modulators induce the expression of detoxification enzymes for their own metabolism, leading to a negative feedback loop. We showed that all seven commercially available polyphenols were able to alter the transcriptional activities of both rat and human AhR. At the onset of ingestion, they are likely metabolized mainly within the gastrointestinal tract in phase I cytochrome P450 and hepatic first-pass to detoxify/eliminate potentially harmful dietary constituents[13].

Moreover, other physiological processes such as immune surveillance and microbiota/host interactions are also controlled by the dietary AhR ligands[6,14–16]. For example, the activation of AhR is essential for maintaining and functioning of intraepithelial lymphocytes and gut ILC22 cells, providing a mechanistic link between dietary components to the innate gut immunity and the microbiota[17–19]. AhR induced by FICZ which is a highly potent endogenous AhR ligand, suppressed IL-6 and claudin-2 expression in a mouse induced colitis model, protecting the intestinal epithelial barrier from IL-6-induced disruption, thus maintaining the intestinal barrier[20].

2. Tissue and species-specific AhR response

The cell response to AhR ligands is tissue- and species-specific. For the groups of POPs and other xenobiotic compounds such as PAHs, the rat AhR was more sensitive than the human AhR. In our study, 16 out of the 29 POPs antagonized the rat AhR in hepatoma DR-H4IIE cells, while only five did in human hepatoma DR-HepG2 cells and seven in human mammary gland DR-T47-D cells. In our study about EDs in drinking water resources, benzo[a]pyrene (BaP) induced a maximum response of 171% of the maximal response induced by TCDD in DR-H4IIE cells, and only 27% in DR-HepG2 cells. In addition, we also saw a synergistic response in DR-T47-D exposed to BaP, 10-fold higher than the cell response to TCDD alone, which was not observed in DR-HepG2 cells. The ED mixture displayed an AhR agonistic effect only in rat DR-H4IIE cells, and not in human DR-HepG2 cells.

The potency of FICZ to activate different AhR isoforms has been shown to be well conserved among various species such as fish[21], frog[22], and birds[23,24]. However, we showed for the first time that the endogenous ligand FICZ was able to activate AhR to a similar extent in rat DR-H4IIE cells and in human DR-HepG2 cells. Moreover, FICZ was more potent than TCDD in

the human cells (40 times more potent than TCDD), while both exhibit a similar potency in the rat cells (after 6h of exposure). As mentioned above, the toxicity of TCDD is probably due to its persistent activity on the AhR, while endogenous ligands such as FICZ will only act transiently. Our results, showing a high FICZ sensitivity and fast clearance rate of FICZ in human DR-HepG2 cells, could mean that FICZ is an essential agonistic ligand for the human AhR. In addition to the duration of AhR activation, the cell types in which the receptor is activated also contributes to the difference in the response of these two ligands[25], which is likely linked to the "quality" of activation through recruitment of various coactivators and transcription factors[26].

We also reported for the first time the species-specific AhR transcriptional activity in cells exposed to different polyphenol groups. Isoflavones (daidzein and genistein) were more active in the rat DR-H4IIE cells, while the flavonol and flavones (quercetin, baicalin, and chrysin) and curcumin strongly affected the human AhR activity in DR-HepG2 cells. Non-flavonoid resveratrol acted equally on both cell lines. It is important to stress here that these activities were only seen after short term exposures (4h or 8h). Since the persistent AhR ligands are more stable and remain longer in the body than natural ligands, depending on the natural ligand levels and the cell context, persistent ligands may have different influences (*e.g.* agonistic-mimicking, antagonistic-blocking or synergism) on the effects of the natural ligands.

3. Risk assessments and mixture effects

3.1. Risk assessment of exposure to mixtures of POPs, EDs, and polyphenols

Our findings showed that the total POP mixture induced significant and dose-dependent antagonistic responses already at concentrations of 125-, 250- and 500-fold blood levels, respectively, in DR-H4IIE, DR-T47-D and DR-Hep G2 cells, although the concentrations of the 29 compounds in this mixture were below their respective lowest observed effect concentrations (LOECs). These levels may realistically be reached after a contamination incident or in exposed populations.

The mixture of ED found in raw water intended for drinking water production in Wallonia was able to activate the AhR transcriptional activity in DR-H4IIE and DR-T47-D cells. The main activity of the ED mixture was due to BaP, which was found in trace amounts. Hence, the possible *in vivo* effect resulting from this ED mixture exposure via drinking water could be minimized by only eliminating this compound from the drinking water.

The polyphenol mixture induced a weak AhR agonistic response relatively similar in both human DR-HepG2 and rat DR-H4IIE cells but it strongly antagonized both FICZ and TCDD AhR transcriptional activation in the human cells with a dose-response curve similar with the one obtained for quercetin. Polyphenols as well as other AhR natural ligands exerted only transient activities due to their rapid metabolic rate. However, in case of continuous or repeated intake of high doses of polyphenols, adverse effects on human health could occur due to the alteration of the AhR transcriptional activity, as polyphenols showed a high antagonistic activity on the human DR-HepG2 cells.

3.2. Predictions of mixture effects

Risk assessment of chemicals in mixture forms is gaining more interest not only in scientific but also in legislation bodies. Albeit acknowledging the threat of human exposure to multiple chemicals, due to a number of challenges, especially the complexity of the problematic formulation and the amount of data involved in the toxicological profiles and exposure patterns, the current risk assessment method mainly relies on a chemical-by-chemical approach with few exceptions (*i.e.* Dioxin TEFs). However, international guidance on mixture risk assessment recently introduced by OECD[27] and EFSA[28], and the Cumulative Risk Assessment (CRA) approach to address the presence of multiple pesticide residues in food, confirms the possibility of risk assessment in case of exposure to mixtures of chemicals.

Our study also contributes to the study of mixture effects, by proposing several modifications of formulas to calculate combined effects and to predict full dose-response curves of mixture effects of chemicals. For the total POP mixture, the best prediction results were obtained using the CA (concentration addition) and GCA (generalized concentration addition) models, a CA derivative. They performed well in predicting $IC_{mix,50}$ = 784-fold blood levels, within a two-fold range from the measured value (374-fold blood levels). In contrast, the IA (independent action) was badly performing to predict the mixture effect of the total POP mixture. On the other hand, IA and CA both performed well in predicting the combined effects of the mixture of seven polyphenols with only a 2-fold overestimation in the dose response curve, probably due to their different mode of actions.

All numerical models (CA, GCA or IA) are approximations, based on the assumption of additivity of the effects or on independent action. Their implementation takes place in order to establish general regulatory guidelines for toxicity assessment of mixtures. While IA seems to violate the systematic instinct of biological organisms by considering strictly independent actions of chemicals in the mixtures, CA (and GCA) is more conservative in assuming strictly

additive effects. The latter have been applied for wider groups of compounds, both with similar and dissimilar mode of actions and the are considered as the “general solution” for risk assessment of mixture toxicity[29].

However, the classical CA model allows only the calculation of effect concentrations (EC or IC) without giving the mixture response or generating the full dose-response curves. In chapter 2, we presented a way to predict the hillslope and maximal response of the mixture from the data of the components, which allowed us to obtain a good predicted dose -response curve. In contrast, both the IA and GCA models allow to generate the full dose-response curve by using only the information from single compounds. However, GCA requires also data from full dose-response curves (the maximum response and the hillslope) of single compounds, which could be sometimes not realistically attained since some compounds may not reach the maximum response in their realistic tested concentration. Therefore, several approaches can be used to predict the mixture effects taking into considerations, all with their pros and cons.

Assessing only the most effective compound(s) could be considered to select the models for mixture predictions and even to perform the calculations. For example,, the antagonism of the total POP mixture was due to chlorinated compounds and, in particular, to PCB-118 and PCB-138, which caused 90% of the antagonistic activity in the POP mixture. The agonistic activity of BaP explained most of the agonistic activity of the total ED mixture in DR-H4IIE cells, while the combined agonistic effects of BaP and bisphenol A explained most of the agonistic effects of the ED mixture in the ER-MMV cells. Similarly, the antagonistic activity of quercetin and resveratrol made up the antagonism of the polyphenol mixture on the DR-HepG2 cells. Because the POPs seem to act in the same mode of actions via competing with TCDD to bind to the AhR, CA and GCA models were able to predict their effects. Meanwhile, the polyphenols (quercetin and resveratrol) seem to affect the TCDD-induced AhR activation in several MOAs, therefore, IA model could also explain the mixture activity. This study suggested that the endocrine disrupting activities of chemicals in human daily life exposure involve more than one mechanism, and that their (anta-)agonistic effects on different receptors, in addition to their potential additive or synergistic effects in the mixture, should be considered in risk assessments.

General conclusions

- **AhR antagonistic activities of the POPs**

- A majority of POPs contaminating human blood (16 out of 29) are AhR antagonists.
- Total POP mixtures relevant for human exposure are AhR antagonistic.

- **Species and tissue-specific AhR responses**

- The rat AhR is more sensitive than the human AhR to POP and isoflavones (daidzein and genistein) exposure.
- The human AhR is more sensitive than the rat AhR to the following ligands: FICZ, the flavonol quercetin and two flavones (baicalin and chrysin), curcumin, and the mixture of the seven polyphenols.
- Benzo(a)pyrene induces a synergistic response in AhR-reporter human mammary gland cells (DR-T47-D) co-exposed to TCDD, inducing a 10-fold higher response than in the cells exposed to TCDD alone.

- **Identifying the actual chemical(s) which are the most active in the mixtures**

- Chlorinated compounds are the drivers of the activity of the total POP mixture, in which PCB-118 and PCB-138 contribute for 90% of the total POP mixture effect.
- Benzo(a)pyrene and bisphenol A contribute the most to the activities of the ED mixture.
- Quercetin and resveratrol cause the antagonism of the polyphenol mixture.

- **Predicting the effect of the mixtures based on the activity of single compounds**

- The dose-response curve of the total POP mixture was predicted successfully by concentration addition and general concentration addition models.
- Both concentration addition and independent action are able to predict the activities of the polyphenol mixture.

Future directions

Mixed exposure is more realistic for chemical exposure in human daily life. Assessing mixture effects is the appropriate approach for risk assessment of chemicals. Our work contributes to go forward in this direction. We could further study the applicability of the models for predicting the combined effects of the real contaminated samples. We have also opened some avenues for future research. For example, the antagonism of the total POP mixture deserves further investigation regarding their associated health effects. The endocrine disrupting activities of the identified chemicals (chlorpyrifos, bisphenol A, fluoranthene, phenanthrene, and benzo(a)pyrene) in raw water should be investigated further *in vivo*. In addition, the potential of using polyphenols (quercetin and resveratrol) for antagonizing dioxin-induced AhR activation could be investigated *in vivo*.

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Annex 1: List of Conferences

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2. **Doan** TQ, Connolly L, Muller M, Scippo ML. The transactivation activity of common used polyphenols on both rat and human aryl hydrocarbon receptor (AhR) in the present and absent of AhR endogenous (FICZ) and xenobiotic (TCDD) ligands. Dioxin, August 2019, Kyoto, Japan.

3. **Doan** TQ, Berntsen HF, Zimmer KE, Verhaegen S, Ropstad E, Connolly L, Muller M, Scippo ML. Exposure to defined mixtures of persistent organic pollutants (POPs) leads to mitochondrial injury in the rat DR-H4IIE cells, using high content analysis screening. Dioxin, August 2019, Kyoto, Japan.

4. **Doan** TQ, Goya-Jorge E, Muller M, Connolly L, Gozalbes R, Scippo ML. In vitro and in silico study of agonistic and antagonistic activities of 29 persistent organic pollutants (POPs) towards the rat and human aryl hydrocarbon receptor (AhR). 20th International Congress on In Vitro Toxicology (ESTIV2018), October 2018, Berlin, Germany

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Annex 2: List of Publications

1. **Doan**, T.Q.; Berntsen, H.; Zimmer, K.; Verhaegen, S.; Ropstad, E.; Connolly, L.; Igout, A.; Muller, M.; Scippo, M. A. Realistic Mixture of Persistent Organic Pollutants (POPs) Inhibits the Transactivation Activity of the Aryl Hydrocarbon Receptor (AhR) in Vitro. Environ. Pollut. 2019, 254, 113098. https://doi.org/10.1016/j.envpol.2019.113098.

2. E. Goya-Jorge, T.Q**. Doan**, M.L. Scippo, M. Muller, R.M. Giner, S.J. Barigye & R. Gozalbes (2020) Elucidating the aryl hydrocarbon receptor antagonism from a chemical structural perspective, SAR and QSAR in Environmental Research, 31:3, 209-226, DOI: 10.1080/1062936X.2019.1708460

3. **Doan**, T.Q.; Connolly, L.; Igout, A.; Muller, M.; Scippo, M. In vitro differential responses of rat and human aryl hydrocarbon receptor to two distinct ligands and to different polyphenols. Submitted to Environmental Pollution.

4. **Doan**, T.Q.; Connolly, L.; Nott, K; Igout, A.; Muller, M.; Scippo, M. In vitro profiling of the potential endocrine disrupting activities affecting steroid and aryl hydrocarbon receptors of compounds and mixtures prevalent in human consumption-relevant water. Submitted to Chemosphere.

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A mixture of persistent organic pollutants relevant for human exposure inhibits the transactivation activity of the aryl hydrocarbon receptor *in vitro*☆

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Generalized concentration addition model

ABSTRACT

While humans are exposed to mixtures of persistent organic pollutants (POPs), their risk assessment is usually based on a chemical-by-chemical approach. To assess the health effects associated with mixed exposures, knowledge on mixture toxicity is required. Several POPs are potential ligands of the Aryl hydrocarbon receptor (AhR), which involves in xenobiotic metabolism and controls many biological pathways. This study assesses AhR agonistic and antagonistic activities of 29 POPs individually and in mixtures by using Chemical-Activated LUciferase gene eXpression bioassays with 3 transgenic cell lines (rat hepatoma DR-H4IIE, human hepatoma DR-Hep G2 and human mammary gland carcinoma DR-T47-D). Among the 29 POPs, which were selected based on their abundance in Scandinavian human blood, only 4 exerted AhR agonistic activities, while 16 were AhR antagonists in DR-H4IIE, 5 in DR-Hep G2 and 7 in DR-T47-D when tested individually. The total POP mixture revealed to be AhR antagonistic. It antagonized EC_{50} TCDD inducing AhR transactivation at a concentration of 125 and 250 and 500 fold blood levels in DR-H4IIE, DR-T47-D and DR-Hep G2, respectively, although each compound was present at these concentrations lower than their LOEC values. Such values could occur in real-life in food contamination incidents or in exposed populations. In DR-H4IIE, the antagonism of the total POP mixture was due to chlorinated compounds and, in particular, to PCB-118 and PCB-138 which caused 90% of the antagonistic activity in the POP mixture. The 16 active AhR antagonists acted additively. Their mixed effect was predicted successfully by concentration addition or generalized concentration addition models, rather than independent action, with only two-fold IC_{50} underestimation. We also attained good predictions for the full dose-response curve of the antagonistic activity of the total POP mixture.

1. Introduction

The aryl hydrocarbon receptor (AhR) was originally characterized as a xenobiotic mediator (Hankinson, 1995). It is often called the "dioxin receptor" as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and several dioxin-like (dl) compounds are AhR agonists. Adverse health effects associated with exposure to these AhR agonists are widely studied, including abnormal reproduction and development, impaired immune system, liver toxicity and cancers (Consultation, 2000).

AhR physiological roles have recently gained more attention since AhR is activated by a wide range of structurally diverse endogenous and exogenous compounds (Denison and Nagy, 2003). Also, AhR-deficient rodents suffer from various physiological defects in the immune system (Harrill et al., 2015), liver (Gonzalez and Fernandez-Salguero, 1998), kidney (Harrill et al., 2015; Harrill et al.,

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2013), cardio-vascular system (Lahvis et al., 2000), urinary bladder (Butler et al., 2012), *etc*. Additionally, AhR homologues are preserved in animal evolution for 600 million years (Hahn et al., 2017), while invertebrate AhR homologues cannot bind dioxin (Butler et al., 2001), indicating that dioxin detection was not the primary role of this protein. Increasing evidence supports important roles of AhR in normal development and homeostasis, while toxicity induced by AhR xenobiotic ligands could be due to perturbation of these normal processes (Marlowe and Puga, 2005).

Upon ligand binding, the AhR is translocated from cytosol into the nucleus where it joins its dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT). This AhR/ARNT complex then binds to a DNA sequence called dioxin responsive element (DRE) to activate the expression of a battery of genes, including both phase I and phase II xenobiotic metabolism enzymes, of which *cyp1a1* is the best characterized. Hence, methods measuring *cyp1a1* gene expression are widely accepted for determining AhR activation (Wall et al., 2015), among which cell-based screening methods such as Chemical-Activated LUciferase gene eXpression (CALUX) are the most common (Murk et al., 1996; Scippo et al., 2004; Goeyens et al., 2010).

Many potential AhR ligands are persistent organic pollutants (POPs). POPs are resistant to degradation and widely distributed in the environment. They can be detected in almost every human sample, including fetuses and embryos (Vizcaino et al., 2014). They tend to bioaccumulate and biomagnify in living organisms, resulting in toxic health effects to both humans and wildlife (Convention, 1997).

Presently, chemical risk assessment mainly relies on a chemical-by-chemical approach (Bopp et al., 2018). In real life, humans are exposed not to an individual POP, but to highly complex POP mixtures (Kortenkamp, 2007). Understanding mixture toxicity is crucial to assess the potential adverse health effects associated with such real life exposure to mixtures of POPs (EC COM, 2012). The mixture effects may be additive, synergistic, or antagonistic depending on whether they are equal, stronger, or weaker than the sum of the effects of individual components, respectively (Berenbaum, 1985). The concepts called “something from nothing” and “a lot from a little” were introduced to describe the mixture effect (Kortenkamp et al., 2009) and proved in a study on fish when a significant effect was observed for a mixture combining individual compounds each at “no observed effect” concentrations (Thrupp et al., 2018).

Due to the various mixture forms and doses, models using the information of components to predict the combined effects are required. Three mathematical models have been developed for this purpose: i) the concentration addition (CA) model was designed for chemicals with similar mode of actions (MOAs) (Loewe and Muischnek, 1926), but has also proven useful for mixtures with dissimilar compounds (Thrupp et al., 2018; Birkhøj et al., 2004; Orton et al., 2014); ii) independent action (IA) (Bliss, 1939), successful in several applications (Backhaus et al., 2004; Payne et al., 2000), applies for chemicals which act independently and have different MOAs; and iii) the generalized concentration addition (GCA) (Howard and Webster, 2009), a CA modified model, was developed for predicting the effects of mixtures containing partial agonists (Howard and Webster, 2009; Howard et al., 2010; Brinkmann et al., 2018).

In an effort to fill the gap in the knowledge of mixture toxicology, a defined mixture of 29 POPs (total POP mixture) was constructed based on their prominence in blood and/or food and breastmilk with the concentration being average blood values from recent Scandinavian studies (Småstuen et al., 2010; Knutsen et al., 2008; Polder et al., 2008) published prior to 2012 (Berntsen et al., 2017). To mimic the exposure of cells (in a tissue) to chemicals that are in the blood stream, reporter gene assays involving cancer cell lines from different tissues (liver and mammary gland) and species (rat and human) were used in this study to determine the AhR transcriptional activities of the selected 29 POPs, individually and in mixtures (the total POP mixture and six sub-mixtures (Berntsen et al., 2017)). The aims were to (a) assess both AhR agonistic and antagonistic activities after exposing the cell lines to the 29 POPs and to the mixtures, (b) identify the main compound(s) responsible for the observed effects of the mixtures, and (c) predict the mixture activity by applying the three available models (IA, CA and GCA).

2. Materials and methods

2.1. Chemicals and suppliers

The total POP and six sub-mixtures were designed and premade by the Norwegian University of Life Sciences, Oslo, Norway as described (Berntsen et al., 2017). The former consists of 29 POPs, where most of them are listed as POPs under the Stockholm Convention on Persistent Organic Pollutants, belonging to three groups: six perfluorinated compounds (PFAAs), seven brominated compounds (BFRs), and 16 chlorinated compounds with seven polychlorinated biphenyls (PCBs) and nine organochlorine pesticides (OCPs). The latter consist of either one single class of compounds (PFAA, Br and Cl) or two combined classes (Cl+Br, Cl+PFAA, Br+PFAA). This way of mixture preparation was to enable the study of the effect of adding or removing one chemical group on different endpoints. As the design of the mixtures was focused on compounds occurring at high concentrations, most dl-PCBs (with the exception of PCB-118) and polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/PCDF) were deliberately excluded. These compounds were also omitted due to their high toxicity at low concentrations in several systems to allow the study of the effect of the non-dl and most prevalent compounds. The components included in the mixtures and their respective concentrations are given in Table S1.

Along with the mixture testing, 29 POPs from the total POP mixture were also examined individually. They were bought from Sigma Aldrich (Missouri, USA) except *o*-chlordane from Toronto Research Chemicals (North York, Canada) and PCB-118 from Dr Ehrenstorfer (Augsburg, Germany). All chemicals were dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Molinons, France), except HCB in hexane (Merck, Massachusetts, USA).

The 29 individual POPs and the mixtures were stored as stock solutions at −20 °C. Working solutions were prepared from the stock solutions to reach the concentrations mentioned in Table S1. The highest tested concentration was 50 μM for all PCBs and PFAAs and 20 μM for BFRs except BDE-100, BDE-153, BDE-154 and BDE-209 (1 μM) due to stocks available. OCPs were tested at the maximum concentration of 100 μM for γ-HCH and dieldrin, or 80 μM for all the others. The concentrations for mixture exposure are presented as “fold blood levels”, relative to the average contaminant levels found in human blood of the Scandinavian population. The total POP mixture and sub-mixtures were tested at concentrations between the estimated concentrations in human blood and maximum 3000 fold blood levels.

2.2. Determination of aryl hydrocarbon receptor agonistic and antagonistic activities

2.2.1. Cell-based assays

Rat and human dioxin responsive (DR) cell lines were used. Rat hepatoma DR-H4IIE cells were from BioDetection System (Amsterdam, The Netherlands) while both human cell lines

(hepatoma DR-Hep G2 and mammary gland carcinoma DR-T47-D) were previously home-made (Liege, Belgium) (Van der Heiden et al., 2009). A vector containing an AhR-controlled luciferase reporter gene was stably integrated into these cells. The vector integrated into DR-H4IIE cells contained four native DREs (from the upstream promotor of the mouse *cyp1a1* gene), leading the MMTV (Mouse Mammary Tumour Virus) promoter (Garrison et al., 1996), while both DR-T47-D and DR-Hep G2 cells were transfected with a vector containing four synthetic DREs regulating the thymidine kinase promoter (Van der Heiden et al., 2009). The cells were routinely cultured in MEMα (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% v/v fetal bovine serum (Greiner, Kremsmünster, Austria), 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma Aldrich, Missouri, USA), and incubated in a H_20 saturated atmosphere containing 5% CO_2, at 37 °C.

The methodology for the DR-CALUX (Dioxin Responsive Chemical-Activated LUciferase gene eXpression) bioassays was described in detail elsewhere (Van der Heiden et al., 2009; Scippo et al., 2005). Briefly, cells were first seeded in white clear-bottomed 96 well microplates (Greiner, Kremsmünster, Austria) and incubated for 24 h to reach about 90% of confluence in the well. After 24-h exposure, the cells were washed with phosphate buffered saline (Sigma Aldrich, Missouri, USA) and treated with lysis solution (containing Triton X100, Sigma Aldrich, Missouri, USA). Luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) were then added to the cell lysate to produce luminescence, which was measured by using a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). The cells were exposed, in triplicates, to a dilution series of the tested compound/mixture in both agonistic and antagonistic tests. For the latter, the cells were co-exposed with a constant concentration of 15 pM, 150 pM and 650 pM TCDD corresponding to TCDD EC_{50} in DR-H4IIE, DR-T47-D and DR-Hep G2 cells, respectively.

In order to verify whether AhR antagonists compete for the same, single site on the AhR with the agonist (TCDD), additional antagonistic tests were performed for selected compounds by co-exposing DR-H4IIE cells to different concentrations of the tested compounds and a constant saturating TCDD concentration (20 nM). Using the agonist (TCDD) at clearly saturating concentrations would make it impossible for a lower affinity antagonist to affect transcriptional activation at all.

All the exposure experiments were repeated at least three times independently. The final concentration of DMSO in the culture medium for the single POPs was 0.2% and 0.3% for agonistic and antagonistic tests, respectively, while they were 0.3% and 0.4% for the mixtures. For quality control, a TCDD reference curve was performed on each plate.

MTT cell viability and LDH cell cytotoxicity were performed along with visual inspection of cell morphology and attachment. The former was carried out in a replicate plate to the DR-CALUX assays. After 24-h exposure, 25 μL MTT dye solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml, Sigma Aldrich, Missouri, USA) was added into each well, followed by a 4-h incubation at 37 °C to form insoluble purple formazan. Then, 100 μL isopropanol (Merck, Massachusetts, USA) was added into the plates to dissolve the formazan for 2 h. The MTT formazan absorbance was read at 550/630 nm by a spectrophotometer (ELx800™ BioTek, Winooski, USA). Because the MTT data need to be interpreted with caution and are not necessarily related to cell death, we performed the LDH cell cytotoxicity as well. The Pierce™ LDH Cytotoxicity Assay Kit was purchased from Thermo Fisher Scientific (Massachusetts, USA) and operated according to the manufacturer's instructions (absorbance at 490/630 nm).

2.2.2. Calculations of EC_{50}, IC_{50} and efficacy (RPC_{Max})

Final results were presented as relative responses, *i.e.* percentages of the cell response to the tested compound/mixture compared to the maximum response of the cells to TCDD on the same plate for agonistic activities, or to spike-in TCDD EC_{50} for antagonistic activities. Dose-response curves were generated by Graphpad PRISM software, version 7 (San Diego, California, USA) by fitting a four-parameter non-linear regression for agonistic (Eq. (1)) or antagonistic (Eq. (2)) tests.

$$Y_{agonistic} = B + \frac{x^H (T - B)}{x^H + EC_{50}{}^H} \quad (1)$$

$$Y_{antagonistic} = B + \frac{T - B}{1 + \frac{x^H}{IC_{50}{}^H}} \quad (2)$$

where x is the concentration of a tested compound/mixture inducing the relative response $Y_{agonistic}$ or $Y_{antagonistic}$. EC_{50} and IC_{50} are the half maximal effective concentration for an agonist and antagonist, respectively (OECD, 2016). B = bottom, T = Top, H = Hillslope.

The lowest observed effect concentration (LOEC) is the lowest tested concentration at which a significant effect ($p < 0.05$) was observed. The maximum observed effect concentration (MOEC) is the lowest tested concentration causing the maximum effect ($p < 0.05$). ANOVA (Graphpad PRISM) was used to determine statistical significance. Prior to ANOVA, tests for homogeneity of variance and normal distribution (transformation if needed) were performed. When no full dose-response curve was achieved, MOEC was considered as the highest concentration of the test series. Efficacy was determined as RPC_{Max} (%), which is the maximum effect induced by the tested compound (OECD, 2016): AhR agonistic RPC_{Max} was the maximum relative response of the compound/mixture compared to the maximum TCDD response, while AhR antagonistic RPC_{Max} was the minimum relative response observed by the maximum inhibition of the test compound/mixture to the spike-in TCDD EC_{50}. The compound/mixture was accepted as active when its relative response was higher than the threshold level $RPC_{Max} \geq 10\%$ for AhR agonists and lower than $RPC_{Max} \leq 70\%$ for AhR antagonists (OECD, 2016).

2.2.3. Calculations of the predicted mixture antagonistic effects

2.2.3.1. Concentration addition (CA). CA model is based on a dilution principle, all the chemicals behave as they are simply the dilution of one another in the mixture. Hence, the effect contribution of one compound to the mixture effect can be totally or partially replaced by the effect of the other. It calculates the effect concentration ($IC_{mix,j}$) of the mixture inducing a specific antagonistic effect j (from 1% to 100%) by considering the concentration partition (p_i) of compound i and its respective effect concentration (IC_{ij}) inducing the same effect j (Eq. (3)). Previously published formulae were adapted (Loewe and Muischnek, 1926; Birkhøj et al., 2004; Payne et al., 2000):

$$IC_{mix,j} = \left(\sum_{i=1}^{n} \frac{p_i}{IC_{ij}} \right)^{-1} \quad (3)$$

The concentration partition p_i can either consider or not the non-active (NA) compounds. Because nonactive compounds do not give IC_{ij}, n is the number of the active compounds.

For each compound i, IC_{ij} inducing the effect j is calculated using its $IC_{i,50}$ and hillslope (H_i) from their fitted curves using Eq. (4) (Graphpad PRISM):

$$IC_{ij} = IC_{i,50}\left(\frac{j}{100-j}\right)^{1/H_i} \quad (4)$$

Because the CA model allows only the calculation of $IC_{mix,j}$, to generate the full dose-response curves, we proposed several possible methods to calculate the hillslope and bottom (H_{mix} and B_{mix}) of the mixture response, while the top was set to 100% as no response.

- the Weighted Mean Hillslope and Bottom (WMHB) (Eq. (5a)) considering p_i to weight the hillslope and bottom of the individual compounds:

$$H_{mix} = \left(\sum_{i=1}^{n} p_i H_i\right)\frac{100}{n} \text{ or } B_{mix} = \left(\sum_{i=1}^{n} p_i B_i\right)\frac{100}{n} \quad (5a)$$

- the Averaged Hillslope and Bottom (AvBH) considering the average of the hillslope and bottom of the individual compounds (Eq. (5b)):

$$H_{mix} = \frac{\sum_{i=1}^{n} H_i}{n} \text{ or } B_{mix} = \frac{\sum_{i=1}^{n} B_i}{n} \quad (5b)$$

- the Formulated Hillslope and Bottom (FoBH) (Eq. (5c)) using the formulae of the CA:

$$H_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{H_i}\right)^{-1} \text{ or } B_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{B_i}\right)^{-1} \quad (5c)$$

2.2.3.2. Independent action (IA). This method assumes that the effect of each component is an independent event (Bliss, 1939). Thus, the probability to exert a specific effect of the mixture is the joint probability of the effect of each compound applied independently. For calculating the relative response of the mixture, the data for individual compounds were converted into a probability.

An antagonistic effect induced by compound i is obtained by subtracting the measured relative response (R_{ik}) from 100% (100% being the relative response of TCDD EC_{50}) and then converted into a probability (scale 0–1, by dividing by 100). The relative response of the mixture (0%–100%) is calculated from the combination of individual probabilities of each compound using the adapted formula (Eq. (6))[28,29]:

$$R_{mix,k} = 1 - \left(1 - \prod_{i=1}^{n}\left(1 - \frac{100 - R_{ik}}{100}\right)\right) \quad (6)$$

At a specific concentration k, $R_{mix,k}$ is the relative response of the mixture; R_{ik} is the relative response of compound i at that concentration k of the mixture, n is the number of the active components.

2.2.3.3. Generalized concentration addition (GCA). GCA (Eq. (7)) assumes that the hillslope for each component is equal to 1 and considers also their RPC_{Max}[31]. It was adapted for AhR antagonistic activity similarly to the IA model by assuming 100% as no effect:

$$R_{mix,k} = 100 - \frac{\sum_{i=1}^{n} \frac{RPC_{Max,i} C_{ik}}{IC_{50i}}}{1 + \sum_{i=1}^{n} \frac{C_{ik}}{IC_{50i}}} \quad (7)$$

where $R_{mix,k}$ is the relative response of the mixture at a specific concentration k, C_{ik} is the concentration of compound i in the mixture at that specific mixture concentration k. $RPC_{Max,i}$ is the maximum effect of compound i and IC_{50i} is the IC_{50} of compound i.

3. Results

In DR-H4IIE cells, while α-chlordane caused cytotoxicity at the highest tested concentration of 80 μM, *t*-nonachlor already did at 62.5 μM in three cytotoxicity tests (data not shown). They were also cytotoxic for the DR-T47-D at lower concentrations of 30 and 30.5 μM, respectively. These cytotoxic concentrations were excluded from the data analyses. None of the other compounds or mixtures induced cytotoxicity at any tested concentration.

3.1. Aryl hydrocarbon receptor activities of the 29 POPs

3.1.1. AhR – mediated agonistic activities

Only four out of the 29 tested POPs presented AhR agonistic activities ($RPC_{Max} \geq 10\%$). BDE-153, PCB-138, and PCB-118 were active in DR-H4IIE, while BDE-99 was active in DR-T47-D. BDE-99 and BDE-154 were able to trigger a weak agonistic activity ($5\% < RPC_{Max} < 10\%$) in DR-H4IIE, as well as γ-HCH in DR-T47-D (Table S2). No agonistic response was recorded in DR-Hep G2 cells for any of the 29 POPs.

3.1.2. AhR – mediated antagonistic activities

Sixteen out of the 29 individually tested POPs displayed AhR antagonisms. No antagonistic activities were observed for any of the PFAAs in all three cell lines. In contrast, in DR-H4IIE cells, AhR antagonistic responses were recorded for 16 POPs including all PCBs, most of the OCPs (except *p,p'*-DDE, α-HCH and β-HCH), and three out of the seven BRFs (BDE-47, BDE-99 and HCBD) (Table 1). PCB-118 and PCB-138 displayed a V-shaped dose-response curve, switching from antagonistic to agonist behavior at concentrations above 3.5 μM and 27.5 μM, respectively. Hence, their IC_{50} values were determined by only the antagonistic part of the curve. The dose-response curves obtained from DR-H4IIE cells co-exposed to TCDD EC_{50} and the 16 AhR antagonistic POPs are shown in Fig. S1 (solid lines) with a detail in Table S3.

DR-Hep G2 cells were less responsive to the POPs than DR-H4IIE, with only five compounds exerting antagonistic activities, namely PCB-28, PCB-118, PCB-138, HCB and BDE-47 (Table 1). PCB-28 was the most potent compound, almost completely abolishing the activity of 650 pM TCDD in DR-Hep G2, displaying an RPC_{Max} of 7.2 ± 3.6% and an IC_{50} of 6.1 ± 1.4 μM. In DR-T47-D cells, seven out of the 29 POPs showed AhR antagonistic activities (PCB-28, PCB-118, PCB-138, HCB, α-chlordane, *t*-nonachlor and γ-HCH). The highest potencies were found for α-chlordane and *t*-nonachlor with RPC_{Max} of 7.4 ± 12.5% and 27.8 ± 3.5%, respectively.

3.1.3. Evaluating competitive inhibition of 16 antagonists in DR-H4IIE

The AhR antagonistic activities were abolished for all compounds (except α-chlordane and *t*-nonachlor with RPC_{Max} of 56.6% and 56.8%, respectively) when co-exposing with excessive 20 nM TCDD, indicating they are possible AhR competitive antagonists (Fig. S1, dashed lines).

Table 1
AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of 16 POPs in DR-H4IIE, DR-Hep G2 and DR-T47-D cell lines (n = 3, 0.3% DMSO).

Compounds	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (μM)	MOEC (μM)	$IC_{50} \pm SE$ (μM)	RPC_{Max} (%)	LOEC (μM)	MOEC (μM)	$IC_{50} \pm SE$ (μM)	RPC_{Max} (%)	LOEC (μM)	MOEC (μM)	$IC_{50} \pm SE$ (μM)	RPC_{Max} (%)
PCB-28	2.5	25	6.8 ± 1.7	36.6 ± 4.3	2.5	25	6.1 ± 1.4	7.2 ± 3.6	3.5	25	11.4 ± 1.5	48.3 ± 5.9
PCB-52	2.5	50*	7.3 ± 1.2	17.1 ± 5.5	–	–	–	–	–	–	–	–
PCB-101	12.5	50*	17.9 ± 3.8	54.3 ± 3.6	–	–	–	–	–	–	–	–
PCB-118	0.5	2.5	0.3 ± 0.05	67 ± 4.7	12.5	25	9 ± 2.7	38.3 ± 16.5	12.5	27.5	13.6 ± 2.4	49.2 ± 4.9
PCB-138	0.5	2.5	0.6 ± 0.07	42.8 ± 2.8	3.5	25	ND	50.2 ± 9.5	12.5	50*	ND	42.6 ± 2.5
PCB-153	0.01	50*	18.5 ± 2.8	16.7 ± 4.6	–	–	–	–	–	–	–	–
PCB-180	1	50*	7.4 ± 3.3	16 ± 1.4	–	–	–	–	–	–	–	–
HCB	0.075	37.5	17.9 ± 11.6	42 ± 8.7	3.75	37.5	4.5 ± 2.3	39.4 ± 12.7	0.075	30	16.4 ± 2.1	51.7 ± 6.7
α-Chlordane	0.4	50*	28.3 ± 3.3	15 ± 1.1	–	–	–	–	10	30	20.2 ± 2.1	7.4 ± 12.5
o-chlordane	20	40*	26.5 ± 19.4	26.3 ± 1.5	–	–	–	–	–	–	–	–
t-nonachlor	25	50*	34.3 ± 1.8	34.2 ± 8.1	–	–	–	–	25	25	16.8 ± 2	27.8 ± 3.5
γ-HCH	0.5	50	27.5 ± 2.7	40.7 ± 3.5	–	–	–	–	50	75	61.2 ± 2.9	65.4 ± 13.7
Dieldrin	6.25	50	22.4 ± 11.4	59.6 ± 1.9	–	–	–	–	–	–	–	–
BDE-47	0.25	20*	3.1 ± 0.5	17.9 ± 2.7	1.25	12.5	ND	55.3 ± 7.2	–	–	–	–
BDE-99	0.25	10	5.2 ± 1.9	36.3 ± 1.5	–	–	–	–	–	–	–	–
HBCD	0.25	15	35.8 ± 63.9**	58.1 ± 3.1	–	–	–	–	–	–	–	–

LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; RPC_{Max}: observed efficacy expressed as a percentage of the cell response exposed to 15 pM, 650 pM and 150 pM TCDD, respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D, corresponding to the MOEC; ND: Not Determined. * Corresponds to the highest tested concentration. ** IC_{50} estimated beyond tested concentrations. -: no response.

3.2. Aryl hydrocarbon receptor activities of the POP mixtures

3.2.1. AhR − mediated agonistic activities

Exposure to the total POP mixture or to the six sub-mixtures described in Table S1 did not induce any significant ($RPC_{Max} \geq 10\%$) AhR agonistic response in any of the cell lines (data not shown), as it could be expected with the exclusion of dl-PCBs and PCDD/F.

3.2.2. AhR − mediated antagonistic activities

The total POP mixture triggered an AhR antagonistic response in all cell lines (Table 2, Fig. 1A). At a concentration in the culture medium corresponding to the blood level, the total POP mixture did not interfere with the response of the cells to EC_{50} TCDD. In contrast, significant and dose-dependent antagonistic responses were already observed at concentrations of 125, 250 and 500 fold blood levels, respectively, for DR-H4IIE, DR-T47-D and DR-Hep G2, although the concentrations of all 29 compounds were below their respective LOEC at these levels or even at 1000 fold blood levels (Table S1). In DR-H4IIE, the POP mixture displayed a significantly high AhR antagonistic efficacy of 52.5 ± 2.1% at 1000 fold blood levels and an $IC_{50} = 374 \pm 52$ fold blood levels, while in both human cell lines, a significant response was observed, but did not reach below 80%, making the calculation of an IC_{50} not possible.

In parallel, six complementary sub-mixtures (PFAA, Br, Cl, Cl+Br, Cl+PFAA, Br+PFAA) were also tested to study the possible interactions between these groups of compounds. Antagonism was seen for all Cl containing mixtures (the total POP, Cl, Cl+Br and Cl+PFAA mixtures) in DR-H4IIE and DR-Hep G2, while only the total POP and Cl+PFAA mixtures showed responses in DR-T47-D (Table 2).

In DR-H4IIE cells, the three Cl containing sub-mixtures and the total POP mixture gave more or less similar responses with $IC_{50} \cong 400-500$ fold blood levels and $RPC_{Max} \cong 50\%$. This indicates that the chlorinated compounds were responsible for the antagonism of all mixtures where they are present. Also, the antagonistic response curve of Cl+PFAA mixture overlapped that of the total POP mixture, which placed below those of the Cl and Cl+Br (Fig. 1B). These observations suggest that the effect of the Cl mixture was somehow enhanced in the Cl+PFAA mixture, resulting in a dose-response curve overlapping that of the total POP mixture.

3.3. Predictions of the rat aryl hydrocarbon receptor antagonistic activities of the total POP mixture and Cl containing sub-mixtures

We evaluated the capacity of the three different mathematical models (concentration addition (CA), independent action (IA), and generalized concentration addition (GCA)) to predict the IC_{50} and dose-response curves of the total POP mixture in the most sensitive cell line, the DR-H4IIE.

3.3.1. Calculation of bottom and hillslope values

Because the 16 rat AhR antagonists contributed only 4.3% for the mass of the total POP mixture, along with considering all of the 29 POPs to calculating p_i, we also considered only the active compounds, subtracting the weight of the non-active compounds (sNA)

Table 2
AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of the total POP mixture and Cl containing sub-mixtures (Cl, Cl+Br, Cl+PFAA) in DR-H4IIE, DR-T47-D and DR-Hep G2 cells (n = 3, 0.4% DMSO).

Mixtures	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (x bl)	MOEC (x bl)	$IC_{50} \pm SE$ (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	$IC_{50} \pm SE$ (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	$IC_{50} \pm SE$ (x bl)	RPC_{Max} (%)
POP	125	1000	374 ± 52	52.5 ± 2.1	500	1000	ND	80.1 ± 5.8	250	1000	ND	86.6 ± 2.2
Cl	250	1000	562 ± 54	53 ± 0.9	250	2000	ND	59 ± 1.6	–	–	–	–
Cl+Br	125	2000	468 ± 38	64.6 ± 1.7	500	1000	534 ± 253	76.1 ± 3.9	–	–	–	–
Cl+PFAA	75	2000	461 ± 78	41 ± 1.3	500	500	243 ± 104	77 ± 3.8	500	500	ND	77 ± 3.8

LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; RPC_{Max}: relative response at MOEC expressed in % of the response of TCDD EC_{50} 15 pM, 650 pM and 150 pM TCDD (respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D) corresponding to the MOEC; x bl: fold blood levels; ND: Not Determined. -: no response.

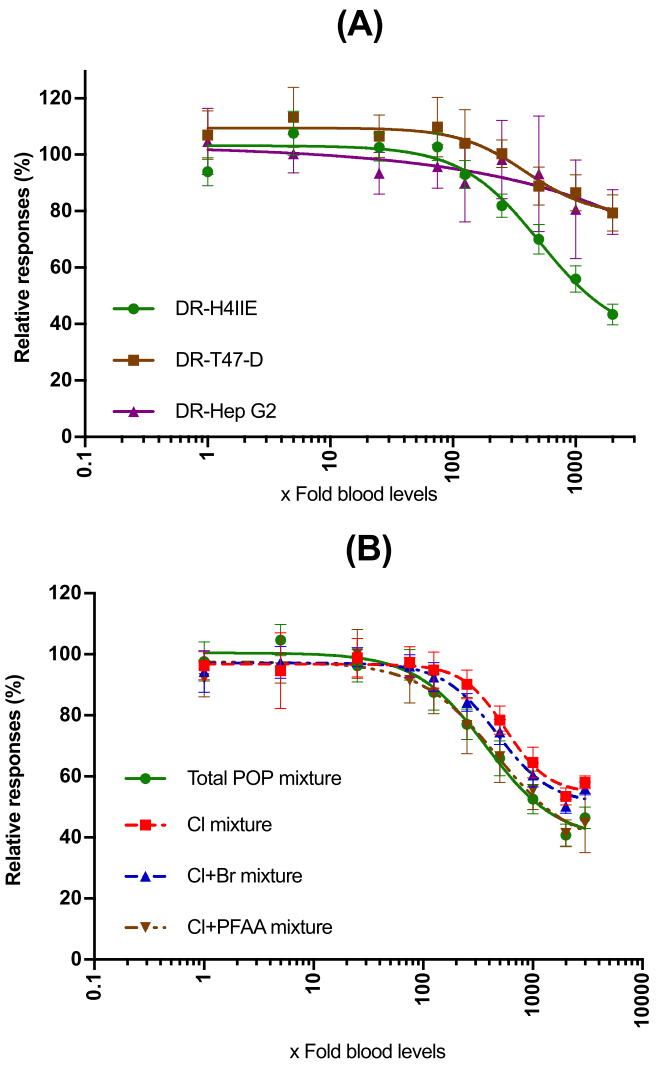

Fig. 1. (A) Dose-response curves obtained from DR-H4IIE, DR-T47-D and DR-HepG2 cells co-exposed to 15 pM, 150 pM and 650 pM TCDD, respectively, and the total POP mixture. (B) Dose-response curves obtained from DR-H4IIE cells co-exposed to 15 pM TCDD, and the total POP mixture, or the Cl, Cl+Br and Cl+PFAA sub-mixtures (Mean ± SD, n = 3, 0.4% DMSO).

as mentioned in section 2.2.3. WMBH method was unable to predict both bottom and hillslope values. AvBH and FoBH showed reasonable predicted values, especially AvBH and sNA FoBH for the total POP mixture and the three Cl containing sub-mixtures (Table S4).

3.3.2. Prediction of mixture effects of the three models

The results obtained after running the three models are shown in Fig. 2. While the IA predicted a really strong response even at the lowest concentrations of the mixture, far out of the range of the measured curve, both the CA (CA-AvBH and CA-sNA FoBH) and GCA predictions resulted in calculated curves comparable to the measured curve. This refers that the 16 active compounds in the total POP mixture acted additively rather than independently.

The measured IC_{50} of the total POP mixture (374 ± 52 fold blood levels) was lower than the predicted value (784 fold blood levels for both the GCA and CA models), while the IA model predicted IC_{50} = 2153 fold blood levels. Thus, both CA and GCA models underestimated IC_{50} of the total POP mixture by about two folds, much less than one order of magnitude.

Concerning the calculated dose-response curves, the two additive models appeared to diverge: GCA produced a somewhat better prediction in the low concentration range, both CA (CA-AvBH and CA-sNA FoBH) closely followed the experimental curve and only diverged at concentrations higher than around 1000 fold blood levels (Fig. 2). Similar predictions were also shown for the three active Cl, Cl+Br, Cl+PFAA sub-mixtures (Table S4, Fig. S2).

3.3.3. Toxic units

Derived from CA model, toxic units (*i.e.* the ratio of the concentration partition of a compound to its IC_{50} ($p_i/IC_{50,i}$)) scales the concentrations of the mixture components to its toxicity, represented by the transcriptional activity of the Cyp1a1 promotor. Thus, it has been applied to identify the main driver(s) for mixture effects in CA model (Loewe and Muischnek, 1926; Backhaus et al., 2004; Evans et al., 2012). Fig. 3A clearly shows that PCB-138 and PCB-118 were the two main contributors for the AhR antagonism of the total POP mixture, constituting to 90% of the total combined activity. Since they were also partial agonists, it is likely that, in the presence of TCDD, they behaved mainly as antagonists especially at low concentrations. Following this prediction, a binary mixture of PCB-138 and PCB-118 was generated according to their concentration in the total POP mixture. The dose-response curve of this mixture followed that of the total POP mixture very closely, with an IC_{50} of 505 ± 67 fold blood levels (while IC_{50} = 374 ± 52 fold blood levels for the total POP mixture) (Fig. 3B).

4. Discussion

4.1. AhR transactivation activities of the 29 POPs and the mixtures

This study shows that a majority of the chemicals composing the realistic total POP mixture are actually AhR antagonists (16 in DR-H4IIE, five in DR-Hep G2 and seven in DR-T47-D cells). As expected, the total POP mixture and the Cl containing mixtures were also shown to be antagonistic. These activities were AhR-dependent, and seemed to act through competition for the TCDD binding site, except for *t*-nonachlor and α-chlordane.

In our study, we tested the AhR transcriptional activity of POPs and POP mixtures using a transcriptional reporter assay, which basically reports the canonical AhR-driven pathways via AhR-ARNT-DRE interactions. However, we observed that two of the compounds (α-chlordane and *t*-nonachlor) do not seem to exert their antagonistic effect through competitive binding to AhR. Several possible non-canonical AhR-driven pathways could contribute to the observed results, such as crosstalk with other nuclear receptors, regulation of cell cycle and MAP kinase cascades, or novel AhR DNA-binding partners (Wright et al., 2017; Jaeger and Tischkau, 2016). Further studies of these mechanisms are required, but were outside of the scope of this paper.

Our results concerning single compound testing are in general consistent with previously published studies, where available. For agonistic activity, PCB-118 displayed highest RPC_{Max} = 61.3% at 50 μM, with EC_{50} = 25 ± 13 μM similar to previously finding (9.3 ± 2.5 μM) (Brenerová et al., 2016). EC_{50} = 4 ± 0.8 μM of BDE-99 was lower than previous report (EC_{50} > 15 μM) (Hamers et al., 2006). For PCB-138, we observed an agonistic effect with a high EC_{50} = 28 ± 6.4 μM, which was not reported before (Brenerová et al., 2016) (Table S2).

For antagonistic activities, in this study, IC_{50} of PCB-28 and PCB-138 were 6.8 ± 1.7 and 0.6 ± 0.07 μM (Table 1), close to previous estimates of 9.0 ± 2.9 μM and 1.4 ± 0.1 μM, respectively (Brenerová et al., 2016). BDE-47 activity (IC_{50} = 3.1 ± 0.5 μM) was similar to

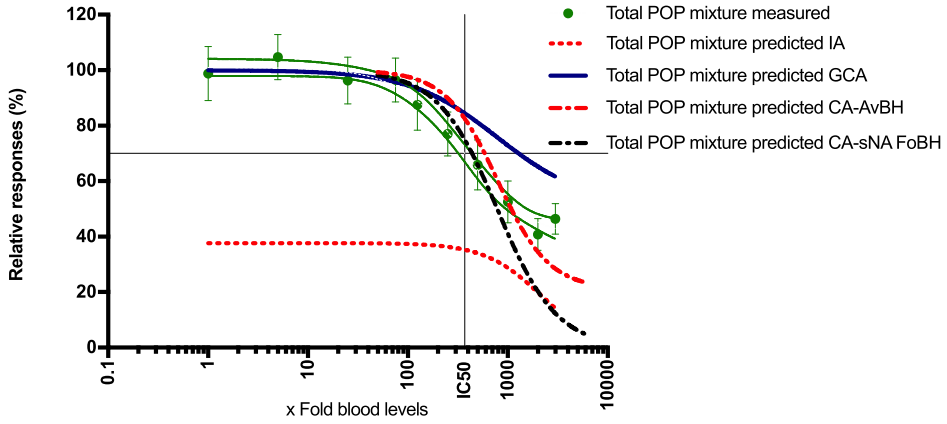

Fig. 2. Measured and predicted dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture, and from three prediction models. CA = Concentration addition, IA = Independent action, GCA = Generalized concentration addition, AvBH = averaged hillslope and bottom, and sNA FoBH = subtracted nonactive compounds, formulated hillslope and bottom. Green dashed lines represent the 95% confidence interval of the measured response.

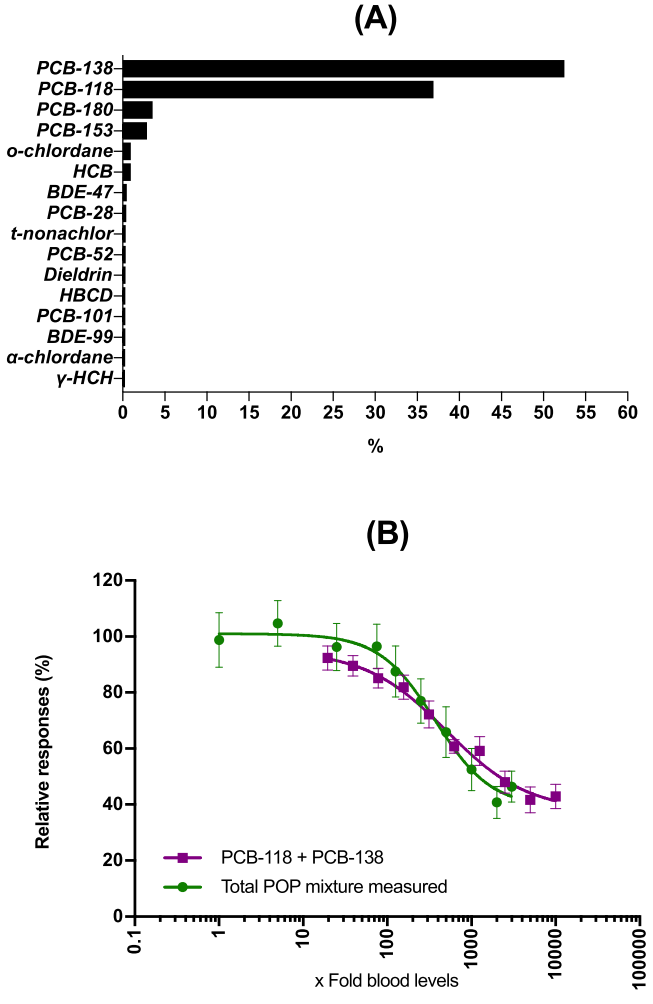

Fig. 3. (A) Distribution of toxic units of the 16 active AhR antagonists. (B) Dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture or a binary mixture consisting of PCB-138 and PCB-118 (Mean ± SD, n = 3, 0.4% DMSO).

those previously reported (2.7 ± 0.7 μM) (Hamers et al., 2006) and (3.7 ± 0.8 μM) (Brenerová et al., 2016). However, the IC_{50} = 5.2 ± 1.9 μM for BDE-99 found in this study was lower than that previously reported (13 ± 0 μM) (Hamers et al., 2006). This study reports for the first-time data for the AhR transcriptional activity of the 29 POPs in both human mammary gland carcinoma DR-T47-D and hepatoma DR-Hep G2 cells.

Differences between IC_{50} or EC_{50} values of this study and those from previous findings (Brenerová et al., 2016; Hamers et al., 2006) may result from differences in the experimental design and the regression methods (*i.e.* the number of concentration points, availability of a maximum effect if a full curve is generated, extrapolation if the maximum effect is not reached, and the regression function used with either four or three parameters). Our study not only confirms and consolidates previous findings (Brenerová et al., 2016; Hamers et al., 2006), but it also contributes new data including full dose-response curves with four parameter fit (see Table S3) which can be used for further data treatment or calculation of the joint effect of any mixture made from these 29 POPs for the rat DR-H4IIE cells.

We observed species and tissue differences in the AhR transcriptional activities of the individual POPs and of the mixtures. In general, the rat DR-H4IIE cells were more sensitive than the two human cells towards the effects on AhR transactivation when exposed to individual POPs or POP mixtures. Several considerations may explain this result. Interspecies differences in AhR structure will obviously shape the sensitivity. Rats are 1000 folds more sensitive to TCDD than guinea pigs (Hulme and Trevethick, 2010). Mouse AhR has a higher affinity than human AhR due to the different position of the important amino acid Valine (V381 in humans corresponding to V375 in mice) (Ramadoss and Perdew, 2004). Human AhR has shown a higher relative affinity for certain structurally compounds *i.e.* endogenous ligands or polyphenols (Flaveny et al., 2009). Moreover, the difference in genetic modification (origins of the integrated promotor and DREs) of the rat DR-H4IIE compared to the two human cell lines could also play a role for the specific responses. Differences in regulatory processes downstream of AhR binding may be responsible, such as differential binding to transcriptional coactivators (Flaveny et al., 2008). Finally, in the antagonistic tests, the POPs have to compete with increasing TCDD concentration (15 pM, 150 pM and 650 pM, respectively for DR-H4IIE, DR-T47-D and DR-Hep G2), which could lead to the lower sensitivity to detect an antagonistic activity of the POPs in the two human cells compared to the rat cells.

4.2. Mixtures relevant for human exposure antagonize AhR activation

The most striking result of this study is that the total mixture of 29 POPs, derived from concentrations found in the blood of a Scandinavian population, and sub-mixtures thereof were found to exert only antagonistic effects on AhR. This observation is consistent with our results obtained from testing each compound alone, revealing a majority of antagonistic compounds. AhR antagonism of POPs has been observed in several screening studies (Brenerová et al., 2016; Hamers et al., 2006) and mixture studies (Harper et al., 1995; Long et al., 2007). However, while the AhR agonistic activity of POPs has been studied for decades, the antagonistic counterpart has not yet received much attention, especially regarding its physiological consequences on an organism's health.

This finding challenges the method of using toxic equivalency factors (TEFs) and toxic equivalent quantities (TEQ) for risk assessments of mixtures of AhR ligands. The World Health Organization assigned TEFs for PCDDs/PCDFs/dl-PCBs, expressed as relative effect potencies compared to the most toxic form TCDD. Regarding their additive mechanism, TEFs are also used to estimate TEQ for a mixture of compounds by adding up the TEF fraction and the concentration of each compound within the mixture (Van den Berg et al., 2006). However, PCB mixtures alone or in combination with PCDDs/PCDFs (usually TCDD) have shown additive but also non-additive responses (Safe, 1998; Safe, 1997). Also, several environmentally abundant biphenyls antagonize the *cyp1a1* induction by TCDD (Wall et al., 2015), while some dl-mono-ortho-substituted PCBs revealed both agonistic and antagonistic properties (Clemons et al., 1998; Hestermann et al., 2000). In many environmental samples, the ratio between PCBs and AhR agonists is above 1000, indicating that antagonisms, resulting from interactions between AhR agonists and PCBs (Fiedler, 2003), are not irrelevant. Therefore, the antagonistic effect of these compounds should be also considered to calculate the effect of mixtures. Since they are more abundant in real-life mixtures, their antagonisms may undermine or even abolish the overall dioxin potency of the environmental mixtures (Wall et al., 2015).

Our finding also raises the issue of the biological significance of a predominantly AhR antagonistic mixture in the blood of a human population. We found antagonistic activities at levels of 125 fold blood levels in rat liver cells or 250 or 500 folds in human mammary gland and liver cells, levels that may realistically be reached after an accident or in exposed populations.

It is important to note that in the total POP mixture used here (Berntsen et al., 2017), no dioxins and dl-compounds were included. That allows us to study the antagonism of the human-exposure relevant POP mixtures by isolating them from the dioxin and dl-compounds. But the roles of the dioxin and dl-compounds in human exposure should have also been considered. Therefore, we attempted to estimate the effect of the total POP mixture in a real-life situation. According to Kvalem et al. (2009), the median of dl-compounds in Norwegian human blood was 33.1 pg TEQ/g lipid, which is equal to 0.6 pM in blood assuming that blood contains 0.6% fat and 1 ml blood = 1 g. Thus, the respective LOECs (for the POP mixture AhR antagonistic activity) in DR-H4IIE, DR-T47-D and DR-Hep G2 of 125, 250, and 500 fold human blood level correspond to 75 pM, 150 pM and 300 pM of dl-compounds, respectively. This concentration is close to the TCDD EC_{50} (15 pM TCDD in DR-H4IIE, 150 pM in DR-T47-D and 650 pM in DR-Hep G2 cells) used in our antagonistic assays. Therefore, it is likely that the total POP mixture would antagonize the activity of these dl-compounds in the Scandinavian population.

Furthermore, the question arises whether an overall AhR antagonistic exposure would actually cause health problems by interfering with the normal AhR function. Increasing evidence suggests that endogenous AhR ligands exist (Rannug et al., 1987; Rannug and Rannug, 2018), complemented by dietary phytochemical-derived AhR agonists/antagonists (Powell and Ghotbaddini, 2014; Jin et al., 2018). AhR induced functions are essential for a variety of normal physiological functions. In mammary tissue, AhR likely plays a physiological role in coordinating development, differentiation, cell growth, and signaling of hormones (Hubbard et al., 2018; Quintana et al., 2008; Roman et al., 2018; Casado, 2016; Hushka et al., 1998). Knock out mice or mice with low affinity AhR variants display impaired survival, growth, fertility, liver function and innate and adaptive immunity (Larigot et al., 2018). It is thus conceivable that the presence of highly stable POPs may interfere with the essential function of AhR controlled by mostly short-lived endogenous and dietary ligands, and thus impair cellular AhR mediated processes. The risk caused by AhR antagonism as the major effect of POPs could well exceed that due to their agonistic effect, however the health effect associated to AhR antagonism is unclear and deserves further investigation.

4.3. Predictions of mixture effects

Risk assessment for mixture exposure is crucial to protect the health of both humans and wildlife. The individual chemical approach underestimates the mixture exposure and decreases the accuracy of risk assessment (Kortenkamp et al., 2009; Thrupp et al., 2018; Silva et al., 2002). In addition, the risk of exposure to multiple chemicals at doses below their threshold, which is the most common case in real-life, should not be underestimated or assumed as no-effect.

In this study, the best prediction results were obtained using the CA (concentration addition) and GCA (generalized concentration addition) models. They performed well in predicting $IC_{mix,50} = 784$ fold blood levels within two folds from the measured value (374 fold blood levels). This is considered as well accepted in predicting the combined effect of complex mixtures *i.e.* the total POP mixture with its components present at low concentration (lower than their LOECs at 1000 fold blood levels) and belonging to different compound groups.

CA is often chosen as the default model (Hardy et al., 2018) for predicting mixture activities, first for mixtures with similar compounds (Loewe and Muischnek, 1926) then expended to dissimilar compounds (Thrupp et al., 2018; Birkhøj et al., 2004; Orton et al., 2014). Previous studies have shown the capability of the CA model to predict the mixture effect using the information of individual chemicals obtained *in vitro* (Birkhøj et al., 2004; Evans et al., 2012; Ghisari et al., 2015; Liu et al., 2015), *ex vivo* (Gaudriault et al., 2017) or *in vivo* (Thrupp et al., 2018; Birkhøj et al., 2004). *In vitro* research has mainly focused on an equimolar mixture with less than ten components and at high exposure concentrations. Birkhøj et al. (2004) successfully applied the CA model to predict the antiandrogenic effect of a mixture of five commonly used pesticides at 10 μM each. In contrast, the CA model was unable to predict the effect on thyroid hormone function and AhR transactivation of another mixture of five different pesticides at the maximum concentration of 50 μM each, due to the presence of an inhibitory compound (Ghisari et al., 2015). Other studies focused on more complex mixtures with multiple components at lower doses, typically below their threshold doses, or on human or environmentally relevant exposure scenarios. Two complex mixtures of 17 estrogenic chemicals were screened for estrogenic activities, reporter-gene (ERLUX) and cell proliferation (ESCREEN) endpoints (Evans et al., 2012). This represents one of the most comprehensive

studies on the effects of mixtures where they were able to predict the effects of the two mixtures.

GCA, on the other hand, has been recently developed and proven useful specifically for calculating mixtures containing partial agonists (Howard and Webster, 2009; Howard et al., 2010; Brinkmann et al., 2018), but has not been applied before to calculate the activity of AhR antagonists. It allows to consider theoretically the fact that some agonists never reach the full activity of TCDD, or that some antagonists present partial agonistic activities.

The difference between CA and GCA models resides in the predicted dose-response curve of the mixture and in the maximum predicted activity of the total POP mixture (Fig. 2). GCA predicting the mixture response, allows to generate the full dose-response curve of the mixture using only the data from testing the individual compounds (*i.e.* RPC_{Max}, concentration and IC_{50}). The reason why this predicted curve diverged from the experimental curve at higher concentrations could result from the assumption that the hillslopes of all components, and so of the POP mixture, are equal to 1, which is clearly not the case (Table S3). However, GCA predicted the bottom ($RPC_{Max} = 52\%$) very close to the observed value for the total POP mixture (52.5%) thanks to its consideration of the RPC_{Max}.

On the other hand, CA provides a prediction of $IC_{mix,j}$ without the full dose-response curve. Therefore, we calculated the hillslope and the bottom values for the mixture response based on its components by formulating several possibilities. The dose-response curve generated by CA with averaged bottom hillslope (CA-AvBH) resulted in the best fit with reasonable hillslope and bottom values (1.7 and 21%) compared to 1.3 and 52.5%, respectively of the measured POP mixture curve. Subtracted non-activated compounds and formulated bottom hillslope (sNA FoBH) predicted an overlapped curve with the observed up to 1000 fold blood levels because of its closer $H_{mix} = 1.5$, but overestimated the extension of antagonism at higher concentration, leading to the prediction of $B_{mix} = 0\%$ for the total POP mixture. B_{mix} is important when predicting the activity of a mixture for risk assessment. Therefore, CA-AvBH rather than CA-sNA FoBH was chosen as a more suitable prediction in our case. The CA model also provides a good prediction of $IC_{mix,50}$ for the response of the DR-H4IIE cells to the active sub-mixtures as to the total POP mixture, and reasonable predicted dose-response curves (Table S4, Fig. S2).

Finally, IA (independent action) was designed specifically for mixtures of compounds with clearly different MOAs to combine probabilities of action of individual compounds. Previous studies showed that IA outperformed CA (Thrupp et al., 2018) or was comparable to CA with equal (Payne et al., 2000) or not more than five-fold differences (Backhaus et al., 2004; Faust et al., 2003) in predicting the combined effects for chemicals having different MOAs. The bad performance of IA to predict either IC_{mix} or the dose-response curve clearly results from the mixture studied here, where we showed that most components act through the same MOAs. At low doses, accumulation of the individual, low probabilities derived for a high number of individual compounds presumably resulted in the dramatic overestimation of the antagonistic effect.

5. Conclusions

We tested the AhR agonistic and antagonistic activities of 29 POPs shown to contaminate human blood, both in individual and mixture forms. AhR transactivation activities in three reporter cell lines exposed to the 29 POPs and the mixtures were different due to the species and tissue-specific responses. The predominant individual activities of the POPs were AhR antagonism, as shown for 16 compounds out of 29 in rat DR-H4IIE cells, and for seven and five compounds in human DR-T47-D and DR-Hep G2, respectively. The total POP mixture already induced a significant AhR antagonistic activity at concentrations of 125, 250, and 500 fold human blood levels, respectively in DR-H4IIE, DR-T47-D and DR-Hep G2, although each individual compound was present at concentrations lower than its LOEC at these levels. Such blood levels of POPs could realistically occur in food or environmental contamination incidents or in highly exposed sub-populations. Chlorinated compounds, among which PCB-118 and PCB-138 contributed 90% to the activity of the total POP mixture, were the drivers for AhR antagonism in DR-H4IIE cells. Finally, CA and GCA proved to be good tools to predict the mixed effect of the total POP mixture with only two-fold underestimated IC_{50} and acceptable does response curves. Hence, the compounds acted additively in the mixtures. Although limitations remain to fully describe the effects of realistic mixtures due to biological complexity, the predictions obtained using CA and GCA seem suitable for establishing general regulatory guidelines for mixture toxicity assessments. In addition, the data generated in this study for individual compounds will be useful to predict the effect of other complex mixtures constituted by these compounds.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113098.

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Elucidating the aryl hydrocarbon receptor antagonism from a chemical-structural perspective

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ABSTRACT

The aryl hydrocarbon receptor (AhR) plays an important role in several biological processes such as reproduction, immunity and homoeostasis. However, little is known on the chemical-structural and physicochemical features that influence the activity of AhR antagonistic modulators. In the present report, in vitro AhR antagonistic activity evaluations, based on a chemical-activated luciferase gene expression (AhR-CALUX) bioassay, and an extensive literature review were performed with the aim of constructing a structurally diverse database of contaminants and potentially toxic chemicals. Subsequently, QSAR models based on Linear Discriminant Analysis and Logistic Regression, as well as two toxicophoric hypotheses were proposed to model the AhR antagonistic activity of the built dataset. The QSAR models were rigorously validated yielding satisfactory performance for all classification parameters. Likewise, the toxicophoric hypotheses were validated using a diverse set of 350 decoys, demonstrating adequate robustness and predictive power. Chemical interpretations of both the QSAR and toxicophoric models suggested that hydrophobic constraints, the presence of aromatic rings and electron-acceptor moieties are critical for the AhR antagonism. Therefore, it is hoped that the deductions obtained in the present study will contribute to elucidate further on the structural and physicochemical factors influencing the AhR antagonistic activity of chemical compounds.

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Introduction

The aryl hydrocarbon receptor (AhR) is an evolutionary conserved transcription factor member of the basic helix–loop–helix (bHLH) family of receptors [1]. The AhR acts as a cytoplasmatic chemical sensor mediating intracellular and cellular signals [2] with its main transcriptional regulatory function being the up-regulation of cytochrome P450 family 1 (CYP1) of metabolizing enzymes [3]. The AhR Ligand Binding Domain (LBD) has been suggested to be one of its two PER-ARNT-SIM (PAS) domains, the PAS-B [4].

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However, comprehensive understanding of the structural and functional profile of the AhR has been limited by the unavailability of an experimentally determined AhR structure co-crystallized with the corresponding functional domains. Consequently, studies aimed at elucidating the interaction modes involved in the AhR signal transduction pathway have relied on homologous systems [5].

AhR owes its first discovery to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is one of the most toxic synthetic compounds known to date and whose effects in biological systems are attributed to the AhR binding and activation [3]. Along with TCDD, several dioxin and dioxin-like compounds have been studied for their AhR agonistic activity and associated toxicological effects [6,7]. However, the ability of AhR to interact with a structurally diverse spectrum of xenobiotic and endogenous ligands suggests that it is highly promiscuous [8].

The induction of the AhR/CYP1A1 axis, as a consequence of AhR agonism, has been reported to enhance oestrogenic detoxification [9], regulate the oxidative balance and to propagate the metabolism of proinflammatory and tumour-promoting metabolites, among other effects [10]. Moreover, AhR plays an important role in the physiological functions of reproductive organs, immune system, liver and vascular development, cardiac function, cell growth, differentiation, homoeostasis and circadian rhythms [2,11–13]. On the other hand, several toxicants and high concern substances have been reported as antagonists of AhR transcriptional activity [7,14]. However, in contrast to AhR agonism, little attention has been paid to the analysis of the biochemical consequences of AhR antagonism and even less on the subsequent toxicological implications [15–17].

Computational models, coupled with in vitro assays, have in the recent times gained increasing utility as alternative tools for providing insight on the pharmacological and/or toxicological effects of chemical compounds [18]. Indeed when proper interpretations are feasible, these techniques jointly provide solid understanding on the structural and functional characteristics relevant for studied bioactivity profiles [19], in addition to their inherent ethical, economic and predictive advantages [20].

It should be noted that most of the AhR computational models reported in the literature have been based on the binding affinity as the endpoint, which is an important drawback since it does not discriminate between agonists and antagonists [21,22]. In addition, as an experimentally determined AhR structure including the respective functional domains is unavailable, in silico initiatives have relied on homology models of the PAS-B domain to provide insights on the possible ligand binding modes [5]. The differences observed in these studies between the agonistic and antagonistic interaction modes suggested greater distortions in the structure of the LBD in case of the latter [5,23]. Nonetheless, little is still known on the chemical structural and functional features that favour the binding of AhR antagonists [24].

Therefore, further analyses of the agonist and/or antagonist responses following AhR binding are needed to gain better understanding of role of the AhR in both toxicological and pharmacological contexts [25]. Certainly, informative experimental bioassays will be necessary to obtain greater insight on the AhR-ligand interaction modes, and particularly to discriminate between agonistic and antagonistic modulations. One of the most popularized assays is the in vitro AhR Chemically Activated LUciferase eXpression (CALUX) bioassay, which has been reported to be useful in the detection of the AhR antagonistic and agonistic effects of dioxin and dioxin-like chemical compounds [26,27]. This assay is

based on the analysis of the induced luciferase response in a recombinant cell line driven by several CYP1A1 dioxin response elements (DREs) as a direct reflection of AhR-mediated transcriptional activity.

The goal of the present manuscript is to analyse the factors, from a chemical perspective, that influence the AhR antagonism of chemical compounds using a combination of in vitro and in silico methods. Firstly, a CALUX reporter gene assay for the AhR antagonistic activity for a set of selected xenobiotics with known toxicity profiles was performed. Then, based on the results obtained from the in vitro assays and an extensive literature review, QSAR and toxicophoric models were built to examine the chemical structural and physicochemical features that modulate AhR antagonism. It should be noted that while QSAR methods have been employed to model and predict the AhR binding capacity [28,29], this is the first time that in vitro, QSAR and toxicophoric approaches are collectively employed to analyse AhR antagonism.

Materials and methods

In vitro evaluation

Chemicals and reagents

A set of 68 chemical compounds was tested in vitro for their AhR antagonistic activity due to their suspected or suggested toxic effects. Twenty-nine of them correspond to Persistent Organic Pollutants (POPs) prevalent in Scandinavian human blood [30] and the remaining 39 corresponded to a chemical library belonging to the Laboratory of Food Analysis of the University of Liège. The tested compounds were mostly obtained from Sigma-Aldrich (Missouri, USA), for details see Supporting Information SI-1. All the chemicals were dissolved in dimethyl sulphoxide (DMSO) (Acros Organics, Molinons, France) as the stock solution of 20 mg/mL and kept in −20°C. The standard solution of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (purity > 98%) in DMSO was supplied from Wellington laboratories (Guelph, Canada). The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Aldrich (Missouri, USA).

AhR CALUX assay

The AhR-CALUX bioassay was developed based on a stably transfected dioxin response (DR) rat hepatoma (H4IIE) cell line, consistent with previous reports in the literature [6,31]. These cells were obtained from BioDetection System (BDS) (Amsterdam, The Netherlands) transfected with four native dioxin response elements from the upstream region of the mouse *cyp1a1* gene leading the Mouse Mammary Tumour Virus (MMTV) promoter and controlling expression of the luciferase gene were stably integrated into the cell's genome. The AhR transactivation activity of the compounds was reported as the expression of the inserted luciferase and measured by light production.

The cells were maintained in *α*-MEM (ThermoFisher, Massachusetts, USA) with 10% v/v foetal bovine serum (Greiner, Kremsmünster, Austria) and 50 IU/mL penicillin and 50 µg/mL streptomycin (Sigma Aldrich, USA) and were incubated at 37°C in a water saturated atmosphere injected with 5% CO_2.

The AhR antagonistic tests for dioxin responsive chemical-activated luciferase gene expression (AhR-CALUX) bioassays were performed as indicated by the provider BDS.

Briefly, after reaching about 90% of confluence in the culture flask, the cells were trypsinized and seeded homogenously in white clear-bottomed 96 well microplates (Greiner, Kremsmünster, Austria). The cells were then incubated for 24 h and afterwards treated with the test compounds for another 24 h. The experiment was terminated by cell lysis using lysis solution containing Triton X100 (Sigma Aldrich, Missouri, USA). After adding luciferin (Promega, Wisconsin, United States) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) to the cell lysate, the plates were read by a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany).

For antagonistic tests, the cells were co-exposed to the test compounds and 15 pM TCDD corresponding to TCDD EC_{50} in DR-H4IIE. All the experiments were repeated at least three times independently. The maximum concentration tested to determine the AhR antagonistic activity was 40 µM with some exceptions (see SI-1), and the final concentration of DMSO in the culture medium was 0.4%. To ensure the adequacy of the test method and provide a basis for comparisons, a reference curve of the positive control TCDD, as well as concurrent negative and solvent controls were added on each plate.

Cell viability assay

An analysis of the MTT cell viability as well as a visual inspection of the cell morphology and attachment were performed to detect the cytotoxic compounds. After exposure to the test compounds, 25 µL of MTT solution 5 mg/mL was added into each well. The plates were then incubated for 4 h at 37°C to allow for the formation of the purple metabolite formazan from the tetrazolium dye MTT via the activity of the mitochondrial succinate dehydrogenase. Later, the formazan crystals were dissolved during 2 h by adding 100 µL of isopropanol. The MTT formazan absorbance was read at 550/630 nm using a microplate spectrophotometer (ELX800^{TM} BioTek Inc., Winooski, USA).

Results for both reporter gene and MTT assays were presented as relative responses, either as the percentage of the cell response to the tested compound compared to the cell response to TCDD EC_{50} on the same plate for CALUX assays, or to solvent control DMSO for MTT test. A compound was considered an AhR antagonist when it was able to reduce the activity of the TCDD EC_{50} from 100% to at least 70%, while reductions in cell population greater than 15% were considered cytotoxic [32].

QSAR models

Based on both the experimental in vitro results obtained herein and those compiled from an extensive review of the literature, binary classifiers of AhR antagonism were built using statistical modelling methods. The molecular characterization was conducted by means of simple and interpretable chemical structural descriptors.

Structures, descriptors and activity

A dataset of 116 chemicals was built to develop the QSAR classification models. This set comprised of 68 compounds tested in the present report for their AhR antagonistic activity and 48 compounds retrieved from literature. From this dataset, compounds with undetermined or inconsistent activity were excluded from the analysis and therefore minimizing potential error sources (for details see SI-1). A binary scale of activity values was considered in the sense that the compounds observed or reported in the literature to

induce the AhR antagonistic effect, based on the AhR-CALUX method, were labelled as active while those that did not induce any effect were considered as inactive.

The calculated molecular descriptors were implemented in-house, based on definitions obtained from DRAGON [33] and PADEL [34] software. Consistent with the parsimony principle, only the simplest characterizations of the molecular structures were considered. A total of 1929 DRAGON descriptors were calculated corresponding to: constitutional descriptors, information indices, functional group counts, atom-centred fragments, 2D binary fingerprints and 2D frequency fingerprints. From this set, constant variables or those with pair-correlations greater than 0.9 were removed, retaining only 171 descriptors. As for the PADEL software, the Estate and MACCS fingerprints were computed yielding 245 descriptors from which 15 constants were removed. Globally, a total of 401 molecular descriptors were retained and subsequently used as input variables for the chemoinformatic modelling.

Variables and QSAR methods

The selection of molecular descriptors for modelling the AhR antagonistic activity was carried out using the information gain filter as provided by IMMAN software [35]. The information gain defined as the reduction in variable entropy (or uncertainty) given that another variable (response variable in this context) is known. This measure allows variables to be ranked based on their capacity to discriminate cases that belong to different classes and consequently filter out informative variables.

A Principal Components Analysis (PCA) was performed on the selected set of variables and 10 principal components (PCs), explaining more than 90% of the total variance, were obtained. Subsequently these PCs were used as input variables for modelling the AhR antagonistic activity. The PCs were rotated using the orthogonal rotation scheme varimax to maximize the sum of variances of the squared loadings. For the PCA the MATLAB software [36] was employed.

Linear Discriminant Analysis (LDA) and Logistic Regression (LReg) methods were employed for the classification model building. The metrics considered to evaluate the performance of the built classification models were the accuracy, precision, sensitivity and specificity (see below) where: TA = true active; FA = false active; TI = true inactive and FI = false inactive.

$$\text{Accuracy} = \frac{(\text{TA} + \text{TI})}{(\text{TA} + \text{FI} + \text{FA} + \text{TI})} \times 100\%$$

$$\text{Precision} = \frac{\text{TA}}{(\text{TA} + \text{FA})} \times 100\%$$

$$\text{Sensitivity} = \frac{\text{TA}}{(\text{TA} + \text{FI})} \times 100\%$$

$$\text{Specificity} = \frac{\text{TI}}{(\text{TI} + \text{FA})} \times 100\%$$

Validation methods and applicability domain

Internal and external validation experiments were designed to assess the robustness and predictiveness of the built classification models. To this end, a cluster analysis was performed to rationally divide the dataset in training and test sets, based on the following procedure: first, with hierarchical clustering (based on the square-Euclidean distance as the similarity criterion) an optimal number of clusters (*k*) was established using the graph of the amalgamation schedule. Then, k-means method was employed to stratify the structures into clusters according to their similarity. An appropriate distribution of active and inactive compounds was considered during the clustering. With this procedure, two subsets of compounds were generated with 75% of the data comprising the training set and the 25% test set. Internal leave-group-out cross validation (25% of the training set was held out for validation for each fold) was performed to check for possible overfitting during the model building procedure. Ten different combinations of training and test sets were created using cluster analysis and a 10-fold external validation was performed to evaluate the average predictive capacity of the models. This approach has been recommended as the most stringent validation protocol for QSAR models [19]. For the cluster analysis, the STATISTICA [37] software was employed.

The applicability domain (AD) of the built QSAR models was established by using the Euclidean distances and Tanimoto coefficients as the structural similarity measures and the k-nearest neighbour algorithm (k-NN) as the feature space approximator. For this AD analysis the AmbitDiscovery software [38] was used.

Toxicophoric modelling

A diverse set of active AhR antagonistic compounds was selected from the dataset built in the present report for mapping the toxicophoric hypothesis. The selection criterion was based on the hierarchical and k-means clustering previously described. Additionally, a Tanimoto matrix score provided by the PubChem [39] platform was considered. For this study, the PHASE module of Schrödinger [40] software was used.

The three-dimensional structures of the selected set of active AhR antagonistic compounds were generated and employed to build toxicophoric models following a ligand-dependent approach exclusively. The mapped space was unrestricted by considering all volumes and shapes, but with locations and orientations in 3D-spaces allowing the enantiomeric discrimination by absolute coordinated distances. All the available features included in PHASE were analysed at distances of 1.8 Å and with a tolerance of 2.0 Å to match the hypothesis. These features were represented in the toxicophoric models as (A) acceptor, (D) donor, (H) hydrophobic, (N) negative ionic, (P) positive ionic, and (R) aromatic ring. The best alignment was searched considering 50 conformations for each compound. The generated hypotheses were accepted if they fitted at least 50% of the training set. Three to seven features were considered as optimum for hypothesis acceptability.

The goodness of fit of the toxicophoric hypotheses was evaluated using the Boltzmann-enhanced discrimination of receiver operating characteristic (BEDROC), which is a generalization of the ROC metric to deal with the early recognition problem. The difference criterion between toxicophoric models was set to 0.5, while the Phase Hypo Score was employed as the scoring function. The mathematical definitions for all

these metrics are detailed elsewhere and the performance is measured from 0–1 values where 1 represents perfect fitness [41].

Finally, an external set of decoy structures was generated using as seeds the diverse set of active AhR antagonistic compounds employed in the toxicophore hypothesis building. For decoy structure generation, the Directory of Useful Decoys (DUD•E) online platform was employed. DUD•E constitutes the largest and most comprehensive public dataset for benchmarking virtual screening programs and was employed here to validate the predictive capacity of the toxicophoric hypotheses. DUD•E is designed to provide sets of structures with similar physicochemical properties but dissimilar topology to the active compounds being, therefore, unlikely binders [42].

Results and discussion

In vitro results

Out of the 68 test compounds evaluated with the AhR-CALUX assay, 24 of these induced AhR antagonistic transactivity, suppressing the cell response to the spike-in control 15 pM TCDD from 100% to at least 70%. Three of these, two insecticide isomers (*α*-endosulfan and *β*-endosulfan) and the fungicide thiram, reduced the cell viability by more than 15%. Moreover, the former two showed cytotoxic effects in more than 60% of the cell population at a concentration of 40 μM while the latter showed a similar effect even at 20 μM. These three chemicals were therefore excluded from the analysis of AhR antagonism (see SI-1). The remaining 41 chemicals did not show any AhR antagonistic potential and were thus considered as inactive.

QSAR modelling

Database of structures and AhR antagonism

A dataset of 116 chemical compounds with defined AhR antagonistic activity profiles based on the DR-H4IIE cell line was constructed. This database contained organohalogenated (chlorinated, brominated, fluorinated), polyaromatic hydrocarbon, flavonoid, quinone, imidazole, bisphenol and pyrethroid chemical classes. Although a few antibiotics and phytochemicals were included, most of the modelled structures are or have been used as industrial chemicals, pesticides, fungicides, algaecides, insecticides, rodenticides and herbicides. Many of these profiled compounds are reported as potential endocrine disruptors or have been already banned due to their toxic spectrum through different mechanisms.

During the construction of the dataset, interest was placed on the homogeneity of the results obtained from the experimental protocols, in order to obtain logical and interpretable conclusions. Therefore, compounds with ambiguous and/or unclear experimental determinations were discarded, especially for those results extracted from literature. Such stringent selectiveness has been strongly supported by several reports in the literature [19].

The hexachlorocyclohexane (HCH) conformational isomers (α-, β-, γ-, δ-HCH) were excluded since they possessed contrasting activity profiles (the descriptors used in the present study are insensitive to stereochemical differences). For the same reason, two

polycyclic aromatic hydrocarbons were excluded. The hexabromocyclododecane (HBCD) in vitro activity was the only one considered, while the rest of the isomers (α-, β-, γ-HBCD) reported in the literature were discarded. Finally, one structural outlier (chlormequat chloride) was removed during the modelling due to disconnected fragments in the molecular structure.

Moreover, BDE-49 (i.e. 2,2′,4,5′-tetrabromodiphenyl ether) [43] and nine polychlorinated biphenyls (PCBs) [7] were excluded as their activity profiles were not clearly defined in the literature (see SI-1). Three cytotoxic compounds in the MTT assay were not considered (see above).

A standard protocol was followed to guarantee the homogeneity of the final dataset presented in this study that included 93 compounds, 40 active AhR antagonists and 53 inactive. Therefore, this dataset may be considered of acceptable quality and is, to the best of our knowledge, the largest for AhR antagonistic activity reported so far.

Selection and transformation of molecular descriptors

The calculated molecular descriptors were all based on simple and interpretable features of the chemical compounds. Due to the large number of generated descriptors, dimensionality reduction was deemed necessary.

From the calculated 401 molecular descriptors, a set of 96 descriptors was retrieved using the aforementioned information gain filter, and subsequently used to generate orthogonal descriptors using the PCA method. Ten PCs were determined to be the optimum number of PCs based on the eigenvalues of the generated covariance matrix and these described 90% of the total variance in the dataset. The matrix of the PCs coefficients obtained for the set of molecular descriptors and the scores for all the PCs are provided as supplementary information (SI-2).

QSAR models obtained

For the QSAR modelling of the AhR antagonistic activity, simple and interpretable classification algorithms were preferred. Hence, five models were selected following commonly established criteria [19], three corresponding to LDA and two corresponding to LReg methods. The training metrics obtained for these models are shown in Table 1 and additional parameters are provided as SI-3. One of the significant advantages offered by these statistical models, compared to most machine learning algorithms such as random forest, support vector machine or artificial neural networks, is the straightforward

Table 1. Evaluation metrics of the QSAR models.

		Accuracy		*Precision*		*Sensitivity*		*Specificity*	
Model[a]	*PCs*	*Train*[b]	*Ext.V*[c]	*Train*[b]	*Ext.V*[c]	*Train*[b]	*Ext.V*[c]	*Train*[b]	*Ext.V*[c]
LDA	10	88.51	81.48	87.02	73.49	88.01	83.56	89.21	79.95
LDA	9	83.02	81.94	87.02	73.58	88.12	83.33	78.11	79.62
LDA	6	78.01	74.07	76.04	65.33	63.23	77.58	89.30	68.95
LReg	7	88.50	73.61	88.51	64.89	88.20	74.78	89.22	72.14
LReg	6	93.51	78.24	87.01	70.32	88.14	74.89	99.03	79.00

[a] QSAR models based on linear discriminant analysis (LDA) and logistic regression (LReg).
[b] Value of the classification metrics obtained during the training of the models (Additional parameters in SI-3).
[c] Average of the classification metrics made during the 10-fold external validation procedure.

interpretation and consequently applicability in the rationalization of the influence of the different chemical structural features on the modelled property [44].

Moreover, both the internal and external validation procedures yielded adequate statistical parameters, demonstrating the robustness and predictive capacity of the five QSAR models obtained herein. Indeed, all the selected models showed good external validation performance, yielding percentages higher than 60% for accuracy, precision, sensitivity and specificity (Table 1).

These models are the first QSARs for AhR antagonistic activity of which we are aware. Previous QSAR models reported in the literature focused on AhR agonism or non-specific AhR-ligand binding [28,45]. Moreover, the models presented herein were built over a diverse dataset of chemicals and were observed to be consistent with the OECD validation principles [46]. Therefore, these models can reliably predict the AhR antagonistic potential of untested chemicals in their AD.

Toxicophoric mapping

Toxicophoric (or Pharmacophoric) models are defined as an ensemble of steric and electronic features that are necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response [47]. Toxicophoric modelling aims to identify a set of features or restrictions in the molecules and/or their receptors that determine their capacity to interact in any mode and consequently cause toxic effects. These models may be built following structure-based or ligand-based approaches depending on whether the information of the target receptor is employed or not [48,49].

Herein, as a fully crystalized structure of the AhR protein (containing the PAS-B ligand binding domain) is not yet available [25], a ligand-based toxicophoric modelling was performed. The cluster analysis performed during the QSAR modelling was employed to select the most dissimilar compounds for the toxicophoric mapping.

Seven most dissimilar structures were selected (Figure 1) and used to explore toxicophoric hypothesis of AhR antagonism. These chemical compounds are considered to be an environmental and health threat and they showed strong antagonistic activity in the in vitro AhR-CALUX assay.

A total of 8 toxicophoric hypotheses were obtained and their performance assessed based on the internal validation enrichment metrics. Consequently, two toxicophoric models were selected as they showed adequate performance and contributed dissimilar structural information.

Figure 2(a,b) show the spatial representations of the selected Toxicophoric-AhR antagonistic hypotheses for the first: Hydrophobic_Hydrophobic_Hydrophobic_ Aromatic Ring (HHHR) and the second: Acceptor_Hydrophobic_Hydrophobic_ Aromatic Ring (AHHR) models, respectively. Both arrangements included a common segment of two hydrophobic constraints and an aromatic ring as critical features. On the other hand, the latter model included an electro-acceptor group while the former possessed an additional hydrophobic constraint.

An external validation of the two selected AhR-antagonistic toxicophoric models was conducted as recommended [42], using a diverse dataset of 350 decoy structures generated from the seven active molecules employed in the hypotheses exploration.

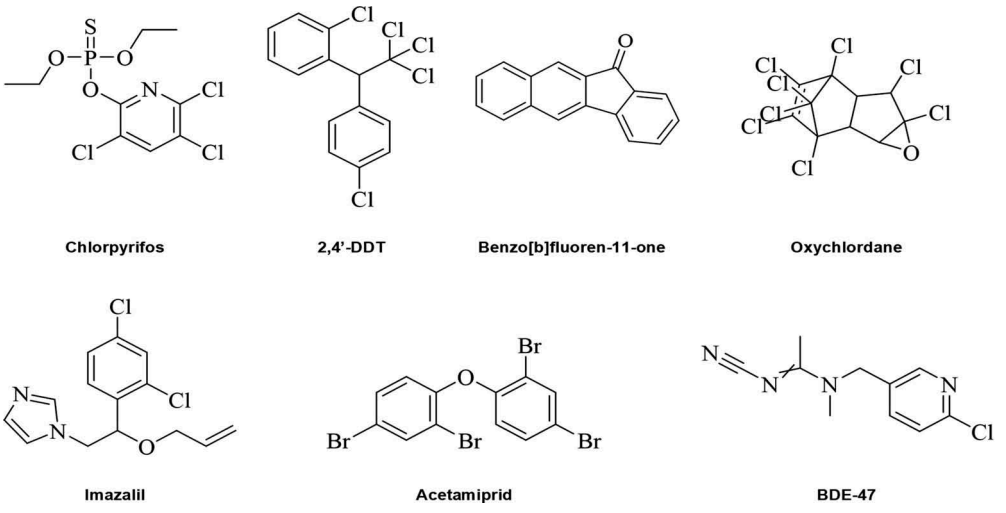


Figure 1. Active structures selected for toxicophoric modelling (For details see SI-1).

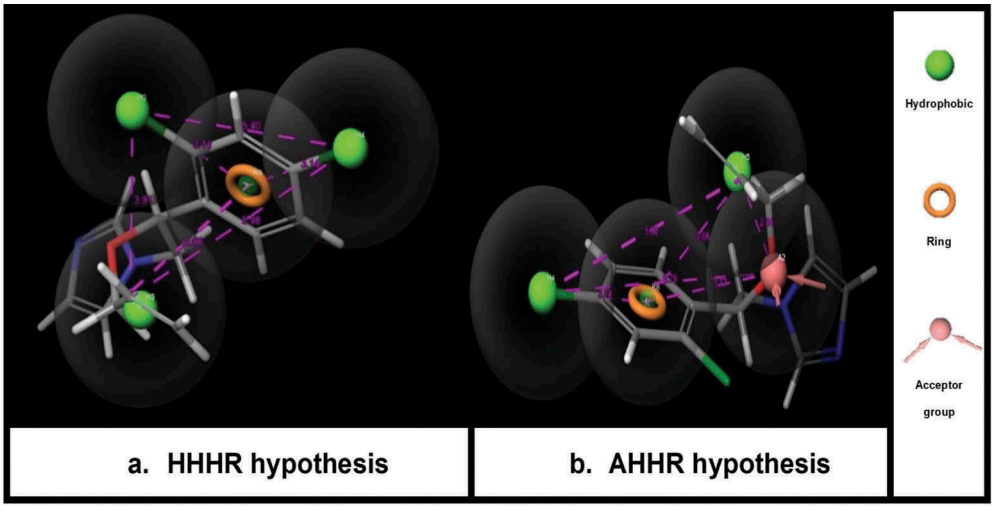


Figure 2. Representation of the toxicophoric models designed in Schrödinger software. Hydrophobic (green-ball), ring (orange ring), acceptor group (pink ball with signalling arrows). a. HHHR: Hydrophobic_Hydrophobic_Hydrophobic_Aromatic Ring. b. AHHR: Acceptor_Hydro phobic_Hydrophobic_Aromatic Ring.

The training and the corresponding external validation results of the HHHR and AHHR toxicophoric hypotheses are shown in Table 2. Overall, the built hypotheses showed good performance as all validation metrics were greater than 0.5 and thus demonstrating their robustness and predictive capacity.

Several pharmacophoric models considering nuclear receptors ERα and ERβ as drug targets [50,51] have been reported in the literature. However, limited distinctions between agonist and antagonistic ligands have been suggested [52]. Furthermore, the few toxicophoric considerations available are generally restricted to drug-safety contexts

Table 2. Results of the training and the external validation for HHHR and AHHR toxicophoric hypotheses.

	Training performance		*External validation*	
Hypothesis	*PHASE*[c]	*BEDROC*[d]	*PHASE*[c]	*BEDROC*[d]
HHHR[a]	0.93	0.68	0.93	0.98
AHHR[b]	0.82	0.69	0.82	0.92

[a] HHHR: Hydrophobic hydrophobic hydrophobic aromatic ring.
[b] AHHR: Acceptor hydrophobic hydrophobic aromatic ring.
[c] Scoring function phase hypo score [41].
[d] Boltzmann-enhanced discrimination of receiver operating characteristic (BEDROC) [41].

[48,49]. Nevertheless, some successful and experimentally validated studies on enzymatic-mediated endocrine disruptive mechanisms have been reported based on the inhibition of 17β-hydroxysteroid dehydrogenases [53].

The first pharmacophoric/toxicophoric estimations based on the blocking capacity of chemicals over AhR are herein proposed. Moreover, considering the good predictive power offered by these computational models, they could be a useful virtual screening tool for chemical compounds with potential AhR antagonistic activity.

Structural and physicochemical interpretations

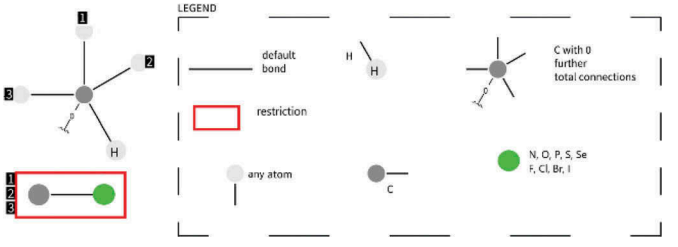
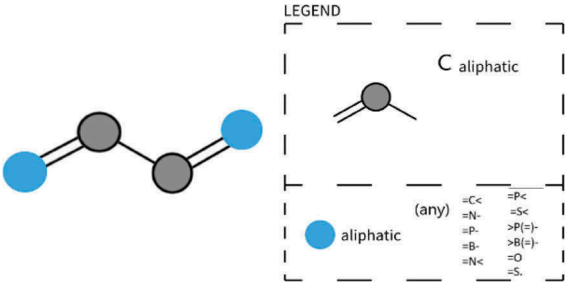
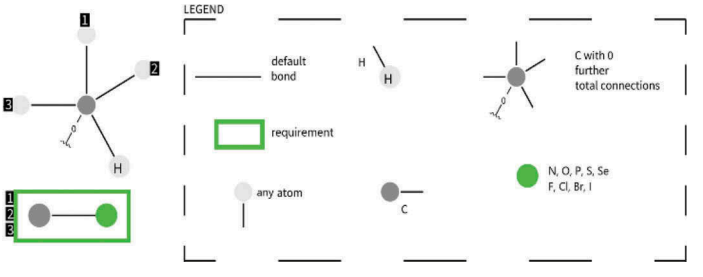
As an essential receptor whose cellular implications are still being discovered, the disruption of the AhR functions could lead to toxic effects that are yet to be fully understood [3,8,11,12]. Hence, the mapping of chemical structural features critical for the AhR agonistic/antagonistic potential could enhance the identification of specific modulators with possible toxic profiles. In this sense, based on the developed QSAR and toxicophore-based models, analyses were performed to further understand how different chemical-structural features influence the AhR antagonistic activity.

The interpretation of computational models is highly recommended by the OECD guidelines to enhance the utility of the QSAR predictive results [46]. Here, only simple 0-2D descriptors were calculated and used for the model building, therefore the elucidation of their contribution to the AhR antagonistic activity in chemical structural terms is straightforward. On the other hand, the toxicophore-based methods provide information on the steric and electrostatic features deemed critical for a given toxicity (or activity) profile. Hence both QSAR and toxicophore models may serve as complementary approaches providing a more complete outlook of the structural and physicochemical factors that influence the AhR antagonistic activity.

The molecular descriptors with relevant contribution were determined based on the PCA factor loadings (SI-2). It has been previously reported that coefficients greater than 0.7 are considered remarkably influential to the generated PCA components [54]. Therefore, the same cut-off was considered to identify the most influential descriptors on the modelled AhR antagonism. Definitions and SMARTS [55] representations of the top five variables obtained are provided in Table 3.

From the toxicophoric models, hydrophobic interactions were identified as an important constraint in active AhR antagonistic molecules. Moreover, the presence of an aromatic ring and an electron acceptor group was suggested as relevant for AhR

Table 3. Molecular descriptors most relevant for the AhR antagonistic activity of the analysed chemical compounds.

ID Code	*Molecular descriptors**
H-046	 H attached to C (sp3) no X attached to next C
nCconj	 Sum of the number of non-aromatic conjugated C(sp2) belonging to any conjugated system, excluding aromatic rings. Including the following atom types: = C<, = N-, = P-, = B-, = N<, = P<, = S<, >P(=)-, >B (=)-, = O, = S
H-052	 H attached to C(sp3) with 1X attached to next C
nBM	Multiple bonds
nCIR	Number of circuits

* Brief definitions/representations of the descriptors using the SMARTs [55] formats of the codified features are provided. The range of values obtained was: H-046 [0–10]; nCconj [0–6]; H-052 [0–12]; nBM [0–26]; nCIR [0–28].

antagonism. These features will now be analysed in detail and the possible complementarity with the QSAR model variables also explored.

Hydrophobic constraints

The hydrophobic features comprising the toxicophoric model and the QSAR model descriptors codifying information on hydrophobicity (i.e. hydrocarbon chains without attached heteroatoms (H-046) and closed hydrophobic circuits (nCIR) of saturated cyclic

hydrocarbons) suggest that non-polar structural segments probably play an important role in AhR-ligand interactions. Indeed, early studies on TCDD where AhR was simply considered as an intracellular, soluble and unknown receptor protein, already recognized its hydrophobic character [56].

Some other reports in the literature have also highlighted the importance of hydrophobicity in AhR-LBD interactions e.g. a strong correlation has been found between the suppression of AhR on TCDD-induced activity and the hydrophobicity of curcumin derivatives [17]. It has also been suggested that the affinity of stilbene derivatives of resveratrol to AhR is enhanced by substituents with high hydrophobicity, among other factors [57]. Moreover, an analysis of the AhR ligand binding pocket revealed that it is comprised predominantly of hydrophobic residues with some polar segments located near the medial positions of the ligands [58]. Therefore, structural fragments with strong hydrophobic character should probably be prioritized in the design of ligands with possible AhR antagonistic activity.

Ring feature

Similarly, both the toxicophoric hypotheses and the QSAR models included ring features [circuit number (nCIR), in the case of the latter] as critical for AhR antagonism. Bearing in mind that the name AhR is attributed to the polycyclic aromatic hydrocarbons (PAHs), which were in fact the very first ligands found to interact with this receptor, it is unsurprising that ring features are suggested to play a critical role in AhR-mediated interactions probably through non-covalent interactions (i.e. between electron-rich and electron-deficient aromatic moieties) [59]. These interactions, also named aromatic donor–acceptor interactions, have been analysed for several decades in the AhR context [60,61]. Firstly, early studies hypothesized that π-π-interactions with phenyl and tyrosyl groups of the AhR protein occurred in the TCDD-mode of action [56]. Also, these interactions have been evoked to rationalize the effect of PBDEs and PCBs upon contact with dissolved organic matter in the environment [28], and have been suggested to play an important role in endocrine disruptive mechanisms e.g. in ER disruption [62].

Nonetheless, it is important to highlight that there ligands without the neutral, hydrophobic and non-polar molecular characteristics of PAHs that have also been shown to have effects on AhR, demonstrating that these interactions depend on the contribution of multiple factors [2,63].

Acceptor-group presence

The charge transfer between molecules and biological systems commonly influences the potential toxicity of the former [64]. Herein, one of the proposed toxicophoric hypothesis suggested the presence of an acceptor group as a relevant structural feature for AhR antagonistic activity. Likewise, the QSAR models comprise various key molecular descriptors that could be related to this toxicophoric feature. For example, heteroatoms attached to sp3 carbons (H-046 and H-052), non-aromatic conjugated systems (nCconj) or multiple bonds (nBM) in general could all contribute to electron deficient fragments in the ligand's structure whose interactions may potentially influence the AhR antagonistic effects.

Altogether, the results obtained herein suggest that the presence of electron acceptor groups, connected ring systems (preferably aromatic or delocalized moieties), and most importantly hydrophobic groups seems to be critical for the potential AhR antagonistic effects.

Conclusions

Structural and physicochemical determinants of the AhR antagonistic capacity were assessed using a combination of in vitro (AhR-CALUX bioassay) and in silico (QSAR and toxicophoric mapping) methods. In the former, several chemical compounds were evaluated, and their antagonistic activity profiles determined. In the latter, QSAR algorithms based on LDA and LReg were obtained and validated. These models were shown to possess adequate robustness and predictive power, based on the quality of the obtained statistical validation parameters. Moreover, toxicophoric models were derived and the structural features potentially favouring the AhR antagonistic potential analysed. Interpretations of the QSAR and toxicophore models revealed that electron acceptor groups, aromatic or delocalized ring systems, as well hydrophobic moieties probably favour the AhR antagonistic activity, demonstrating the complementarity of the two approaches notwithstanding their dissimilar conceptual basis. The models proposed herein could be useful in the prediction of the AhR antagonistic capacity of chemical compounds with toxicological and pharmacological applications.

Disclosure statement

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